NTP REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDY OF BENZENE

(CAS NO. 71-43-2)

IN GENETICALLY MODIFIED HAPLOINSUFFICIENT $p16^{Ink4a}/p19^{Arf}$ MICE

(GAVAGE STUDY)

Scheduled Peer Review Date: August 28, 2006

NTP GMM 8

NIH Publication No. 06-4425

National Toxicology Program

National Institutes of Health
Public Health Service
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Genetically Modified Model (GMM) Report series began in 2005 with studies conducted by the NTP. The studies described in the GMM Report series are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected agents in laboratory animals that have been genetically modified. These genetic modifications may involve inactivation of selected tumor suppressor functions or activation of oncogenes that are commonly observed in human cancers. This may result in a rapid onset of cancer in the genetically modified animal when exposure is to agents that act directly or indirectly on the affected pathway. An absence of a carcinogenic response may reflect either an absence of carcinogenic potential of the agent or that the selected model does not harbor the appropriate genetic modification to reduce tumor latency and allow detection of carcinogenic activity under the conditions of these subchronic studies. Substances selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in NTP GMM Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection *per se* is not an indicator of a substance's carcinogenic potential.

The NTP conducts its studies in compliance with its laboratory health and safety guidelines and FDA Good Laboratory Practice Regulations and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. Studies are subjected to retrospective quality assurance audits before being presented for public review.

NTP GMM Reports are indexed in the NIH/NLM PubMed database and are available free of charge electronically on the NTP website (*http://ntp.niehs.nih.gov/*) or in hardcopy upon request from the NTP Central Data Management group at *cdm@niehs.nih.gov* or (919) 541-3419.

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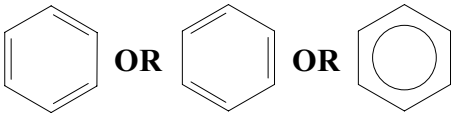
CONTENTS

ABSTRACT



BENZENE

CAS No. 71-43-2

Chemical Formula: C_6H_6 Molecular Weight: 78.1

Synonyms: (6)-Annulene; benzol; benzole; benzolene; bicarburet of hydrogen; carbon oil; coal naphtha; cyclohexatriene; mineral naphtha; motor benzol; phene; phenyl hydride; pyrobenzol; pyrobenzole

Benzene is used primarily as a solvent in the chemical and pharmaceutical industries, as a starting material and intermediate in the synthesis of numerous chemicals, and in gasoline. The major United States source of benzene is petroleum. Benzene has been previously evaluated in 2-year carcinogenicity studies by the National Toxicology Program (1986). In this study, the carcinogenic effects of benzene were studied in the haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse model as part of an ongoing NTP effort to seek improved model systems for toxicology and carcinogenesis studies, especially those that can provide mechanistic information relative to understanding an agent's mode of action. Male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice were administered benzene (greater than 99% pure) by gavage for 27 weeks. Genetic toxicology studies were conducted in mouse peripheral blood erythrocytes.

27-Week Study in Mice

Groups of 15 male and 15 female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice were administered 0, 25, 50, 100, or 200 mg benzene/kg body weight in corn oil by gavage 5 days per week for 27 weeks. All animals survived until the end of the study except one male administered 200 mg/kg. Mean body weights of males administered

50 mg/kg or greater were generally less than those of the vehicle controls throughout the study, and those of 25 mg/kg males were less after week 13. Mean body weights of 200 mg/kg females were less than those of the vehicle controls after week 17. Treatment-related clinical findings in 25 mg/kg or greater males and 50 mg/kg or greater females included black, brown, or gray discoloration (pigmentation) of the feet. The thymus weights of all dosed groups of males were significantly decreased. At weeks 13 and 27, a dose-related decrease in the erythron occurred in males and females. The erythron decrease was shown by decreases in the hematocrit, hemoglobin, and erythrocyte count values in all dosed males and in the 100 mg/kg or greater females. Decreased leukocyte counts, primarily lymphocyte counts, resulted in a dose-related leukopenia in males and females. In males, segmented neutrophil counts were also decreased.

The incidence of malignant lymphoma was significantly increased in 200 mg/kg males compared to the vehicle controls.

In the bone marrow, significantly increased incidences of minimal to mild atrophy occurred in the 100 and 200 mg/kg males compared to the vehicle controls. In the spleen, there were significantly increased incidences of lymphoid follicle atrophy in 100 and 200 mg/kg male mice. The incidence of hematopoietic cell proliferation was significantly increased in 200 mg/kg males. The incidences of atrophy of the thymus in the 100 and 200 mg/kg males were significantly greater than those in the vehicle controls. In the lymph nodes, significantly increased incidences of atrophy (mandibular, mediastinal, and mesenteric) occurred in 100 and 200 mg/kg males, and the incidence of atrophy of the mediastinal lymph node was significantly increased in the 100 mg/kg females. The incidences of skin pigmentation were significantly increased in all dosed groups of males and in 50 mg/kg or greater females.

GENETIC TOXICOLOGY

The frequency of micronucleated erythrocytes was assessed at four timepoints during the 27-week study. Significant increases in micronucleated cells were observed at all timepoints, and the magnitude of the response correlated with duration of dosage.

CONCLUSIONS

Under the conditions of this 27-week gavage study, there was *clear evidence of carcinogenic activity* of benzene in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice based on the occurrence of malignant lymphoma. There was *no evidence of carcinogenic activity* of benzene in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ female mice administered 25, 50, 100, or 200 mg/kg.

Treatment of male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice with benzene was associated with toxicity to the hematopoietic system, lymphoid atrophy, and the accumulation of pigment in the extremities.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 9.

Summary of the 27-Week Carcinogenesis and Genetic Toxicology Studies of Benzene in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice

	Male	Female
Concentrations in corn oil by gavage	0, 25, 50, 100, or 200 mg/kg	0, 25, 50, 100, or 200 mg/kg
Body weights	Dosed groups less than the vehicle control group	200 mg/kg group less than the vehicle control group
Survival rates	15/15, 15/15, 15/15, 15/15, 14/15	15/15, 15/15, 15/15, 15/15, 15/15
Nonneoplastic effects	Bone marrow: atrophy (0/15, 0/15, 0/15, 10/15, 12/15); hemosiderin pigmentation (1/15, 13/15, 13/15, 15/15, 14/15) Spleen: hematopoietic cell proliferation (0/15, 1/15, 0/15, 2/15, 8/15); lymphoid follicle atrophy (0/15, 0/15, 0/15, 15/15, 14/15) Thymus: atrophy (0/15, 0/15, 0/15, 7/15, 13/15) Lymph node (mandibular): atrophy (0/15, 0/15, 0/15, 6/14, 11/14) Lymph node (mediastinal): atrophy (0/11, 0/11, 2/12, 6/9, 6/7) Lymph node (mesenteric): atrophy (1/15, 2/15, 2/14, 13/15, 13/15) Skin: pigmentation (0/15, 15/15, 15/15, 15/15, 14/15)	Lymph node (mesenteric): atrophy (0/15, 2/15, 3/15, 8/15, 6/15) Skin: pigmentation (0/15, 1/15, 8/15, 15/15, 15/15)
Neoplastic effects	Malignant lymphoma: (0/15, 0/15, 0/15, 0/15, 5/15)	None
Level of evidence of carcinogenic activity	Clear evidence	No evidence
Genetic toxicology		
Micronucleated erythrocytes		
Mouse peripheral blood *in vivo:*	Positive in males and females at the 6.5-, 13-, 19.5-, and 27-week sampling times with the magnitude of the response increasing with increasing duration of treatment.	

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results **(clear evidence and some evidence);** one category for uncertain findings **(equivocal evidence);** one category for no observable effects **(no evidence);** and one category for experiments that cannot be evaluated because of major flaws **(inadequate study).** These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as “were also related” to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as “may have been” related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Report on benzene in August 28, 2006, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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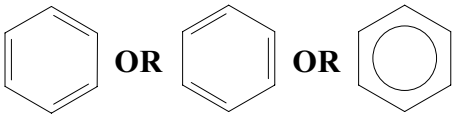
SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

NOTE: A summary of the Technical Reports Review Subcommittee's remarks will appear in a future draft of this report.

INTRODUCTION



BENZENE

CAS No. 71-43-2

Chemical Formula: C_6H_6 Molecular Weight: 78.11

Synonyms: (6)-Annulene; benzol; benzole; benzolene; bicarburet of hydrogen; carbon oil; coal naphtha; cyclohexatriene; mineral naphtha; motor benzol; phene; phenyl hydride; pyrobenzol; pyrobenzole

CHEMICAL AND PHYSICAL PROPERTIES

The representation of the benzene molecule has evolved from the resonance ring structures described by Kekule in 1865 to the structure in which all six carbon-to-carbon bonds are identical (see above representations; NTP, 1986).

Benzene at room temperature is a highly flammable, colorless, transparent liquid having a boiling point of 80° C and a melting point of 5.5° C (NTP, 1986; *Merck Index*, 1996). Slightly soluble in water (1.8 g/L), benzene is miscible with a variety of organic solvents and with oils. Benzene has a vapor pressure of 13 mm Hg, a flashpoint of –11° C, and a flammability limit in air of 1.5% to 8.0%. It is generally available in three grades: refined, nitration, and industrial (NTP, 1986). The differences are based on the content of nonaromatics specified (less than 0.15% for refined) and on the distillation range (less than 1° C for refined and nitration grades and less than 2° C for industrial grade).

Production, Use, and Human Exposure

Benzene is used primarily as a solvent in the chemical and pharmaceutical industries, as a starting material and intermediate in the synthesis of numerous chemicals, and in gasoline (NTP, 2004). The major United States source of benzene is petroleum. In 1994, benzene ranked 17th in production volume among chemicals produced in the United States. In 2002, 7.2 million metric tons of benzene were produced in the United States, and over 4 billion liters were imported.

The primary route of benzene exposure is by inhalation (NTP, 2004). Auto exhaust and industrial emissions account for ~20% and cigarette smoke for ~50% of human exposure (Powley and Carlson, 1999). Occupational exposure affects over 200,000 people per year (ATSDR, 1997). The major industries using benzene are those involved in rubber, paint, shoes, lubricants, dyes, detergents, drugs, and pesticides (Bauer *et al.*, 2003).

Metabolism, Absorption, and Distribution

Mice, rats, rabbits, and humans generally metabolize benzene in a similar manner, although quantities of any one metabolite may vary by species as determined using microsome preparations from these different species (Powley and Carlson, 1999).

Metabolism of benzene by the cytochrome enzymes to phenol, catechol, and hydroquinone, as well as enzyme systems that result in ring-opened forms of benzene may all be involved in producing carcinogenic moieties of benzene (USEPA, 1998). Benzene is metabolized to benzene oxide by a cytochrome P450 multifunctional oxygenase system (CYP2E1 and CYP2B1) primarily in the liver; however, other organs may be involved (e.g., lung) (Powley and Carlson, 1999; Ross, 2000). Benzene oxide is then nonenzymatically transformed to phenol or ring-opened muconaldehyde. Muconaldehyde is metabolized to muconic acid. Phenol is further metabolized (via CYP450) to hydroquinone and catechol. Hydroquinone and catechol can then be metabolized in bone marrow by myeloperoxidase to form *p*-benzoquinone and *o*-benzoquinone (Powley and Carlson, 1999) (Figure 1).

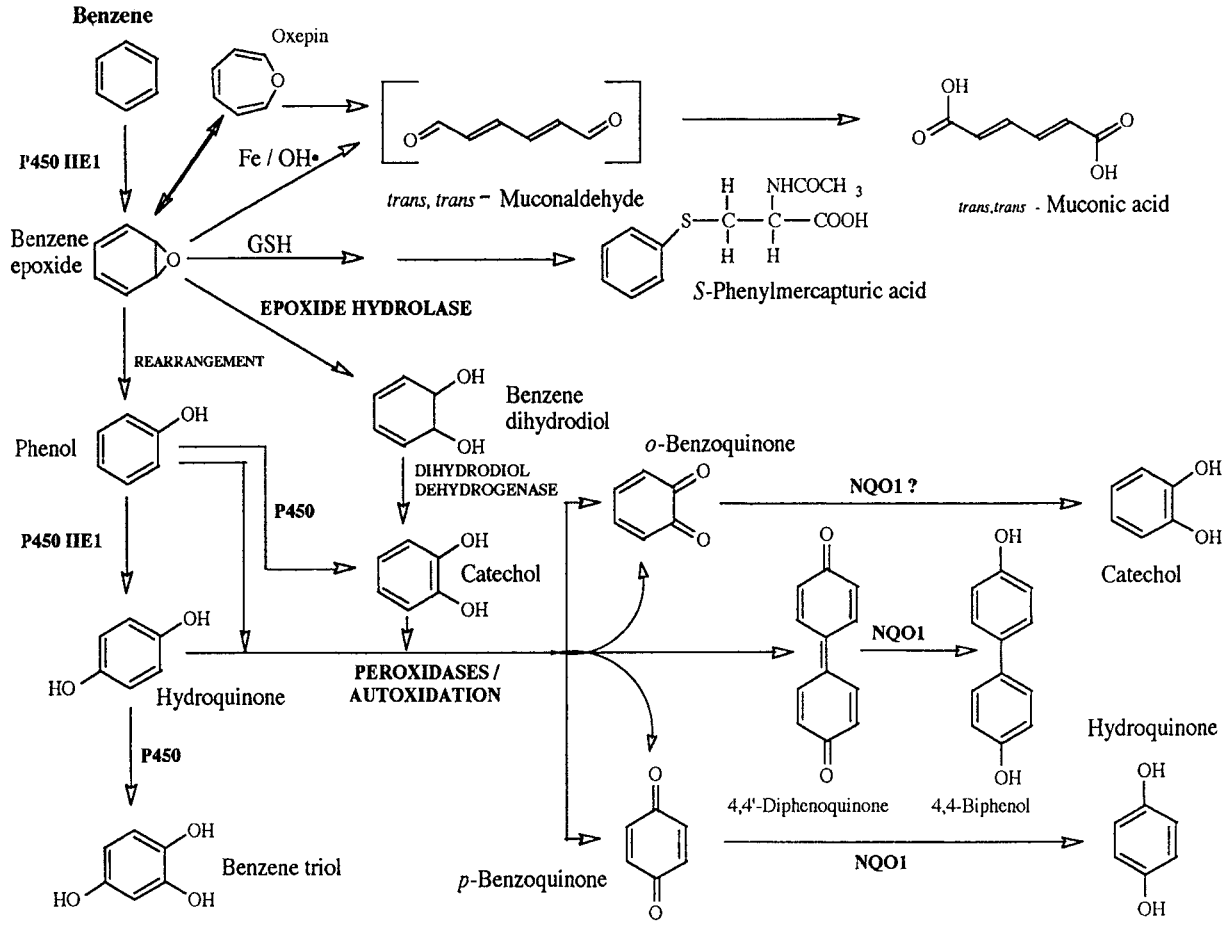


FIGURE 1
Pathways in Benzene Metabolism
Glucuronidation and sulfation pathways have been omitted for clarity (Ross, 2000)

The content of peroxidases, which activate phenols to toxic quinones and free radicals, and sulfatases, which remove conjugated sulfate and thus reform free phenol, may be present at different levels in various organ systems and may help explain the different organ carcinogenic properties of benzene (USEPA, 1998). Polymorphisms in enzyme systems involved with benzene metabolism (e.g., epoxide hydrolase) may affect the amount of metabolite formed, and thus, the ultimate toxicity/cancer effects of benzene (Bauer *et al.*, 2003).

Detoxification reactions include glutathione conjugation of benzene oxide, and sulfate and glucuronide conjugation of phenol, hydroquinone, catechol, and trihydroxybenzene. Following conjugation, metabolites are excreted in the urine (Powley and Carlson, 1999).

Several of the benzene metabolites may contribute to the toxic and carcinogenic effects of the chemical. Some studies suggest that *p*-benzoquinone is responsible for the carcinogenic effects of benzene (Irons, 1985; Powley and Carlson, 1999). Catechols and quinone metabolites can be oxidized and react with DNA to form adducts, leading to mutations and cancer (Cavalieri and Rogan, 2004; Gaskell *et al.*, 2005). Benzene metabolites may also form adducts with proteins or interfere with gap-junction intercellular communication, which may also be involved in benzene toxicity (Rivedal and Witz, 2005; Waidyanatha and Rappaport, 2005). Other studies suggest that nitric oxide can react with benzene metabolites in the bone marrow to form nitro metabolites (nitrobenzene, nitrobiphenyl, and other nitrophenol isomers), and these metabolites may contribute to benzene toxicity (Chen *et al.*, 2004).

Disposition and metabolism studies of C^{14}-labeled benzene have been conducted in male C57Bl/6N mice after a single oral dose of 10 or 200 mg/kg (McMahon and Birnbaum, 1991). In 3-month-old mice, 95% of the labeled 10 mg/kg dose was eliminated in the urine within 48 hours; at 200 mg/kg, 42% was eliminated in the urine and 56% as volatiles in expired air. The major urinary metabolites were hydroquinone glucuronide, phenylsulfate, and muconic acid.

After an oral dose of benzene (0.1 mg/kg) to male Tg.AC mice, male $p53^{+/-}$ mice, and their respective parent strains (FVB/N and C57BL/6), the major route of excretion was again in the urine (Sanders *et al.*, 2001). The major metabolites recovered in the urine were hydroquinone glucuronide, phenylglucuronide, phenylsulfate, and muconic acid.

After an oral benzene dose of 1, 10, or 200 mg/kg to male F344/N rats and male B6C3F$_1$ mice, benzene metabolites were found in blood, urine, liver, lung, and bone marrow (Sabourin *et al.*, 1989). The major metabolites in bone marrow were phenylsulfate and prephenylmercapturic acid, muconic acid, hydroquinone monosulfate, and phenylmercapturic acid in rats and hydroquinone, phenylsulfate, and phenylmercapturic acid in mice.

Differences in the rates of benzene metabolism were observed between male and female B6C3F$_1$ mice after an inhalation exposure to benzene (400 to 2,800 ppm) (Kenyon *et al.*, 1996). Pretreatment of male mice with acetone to induce CYP2E1 enhanced the rate of benzene oxidation. The authors concluded that oxidative metabolism of benzene occurs at a faster rate in males than in females, possibly explaining why more genotoxic damage occurs in males than in females.

TOXICITY

Experimental Animals

The following oral LD$_{50}$ values have been reported for benzene: 0.93 g/kg for Sprague-Dawley rats (Cornish and Ryan, 1965), 3.8 mL/kg for young adult male Sprague-Dawley rats (Kimura *et al.*, 1971), and 5.6 g/kg for male Wistar rats (Wolf *et al.*, 1956). The LC$_{50}$ value for a 7-hour exposure is 10,000 ppm for mice (Svirbely *et al.*, 1943).

Benzene toxicity studies have been summarized by the Agency for Toxic Substances and Disease Registry (2005) and the United States Environmental Protection Agency (2002). Benzene, which is particularly toxic to bone marrow, induces hematotoxicity including leukopenia, lymphocytopenia, granulocytosis, anemia, and reticulocytosis. Lymphocyte counts appear to be depressed sooner and more severely than other cell types, and granulocytes may be the most resistant (USEPA, 2002).

In an NTP (1986) study on the toxic and carcinogenic effects of benzene, F344/N rats and B6C3F$_1$ mice of both sexes were administered benzene by gavage, 5 days per week for 103 weeks. Male rats (50/group) were administered 0, 50, 100, or 200 mg/kg, and female rats and male and female mice (50/group) were administered 0, 25, 50, or 100 mg/kg. Blood was drawn at 12, 15, 18, 21, and 24 months. This study identified a lowest-observed-adverse-effect level (LOAEL) of 25 mg/kg for leukopenia and lymphocytopenia in female F344/N rats and male and female B6C3F$_1$ mice and of 50 mg/kg in male F344/N rats. These were the lowest doses tested, and therefore, a no-observed-adverse-effect level (NOAEL) was not identified.

Humans

Chronic exposure to benzene can result in anemia, thrombocytopenia, leukopenia, and/or aplastic anemia (Robles, 1998) and myelogenous leukemia (Powley and Carlson, 1999). The toxicity of benzene is related to its metabolism. Phenol, hydroquinone, catechol, and benzene oxide metabolites may all be involved in benzene toxicity (Ross, 2000).

Human exposure to benzene may be associated with bone marrow depression, as evidenced by anemia (decreased red blood cell count), leukopenia (decreased white blood cell count), and/or thrombocytopenia (decreased platelet count).

Reproductive and Developmental Toxicity

Experimental Animals

Administration of benzene has been associated with reproductive and developmental toxic effects in a wide range of experimental animals including mice, rats, and other rodents. A complete listing of individual benzene reproductive toxicity studies can be found in the USEPA (2002) report. Benzene is lipophilic and can readily pass the placental barrier and affect embryonal cells directly. Phenol, a major metabolite of benzene, inhibits DNA synthesis in bone marrow and can pass the placental barrier. Benzene may also damage maternal circulation and cause bone marrow depression, resulting in adverse nutritional conditions for the fetus (USEPA, 2002).

Humans

There have been no definitive studies on the effects of benzene on human reproductive capacity, primarily because populations exposed to benzene also are exposed to other chemicals (USEPA, 2002). In industrial settings where benzene exposure occurs along with other chemical exposures, there has been some evidence in female workers for hypermenorrhea, ovarian hypofunction, or disturbances in the menstrual cycle.

CARCINOGENICITY

Experimental Animals

Benzene was a multisite carcinogen in 2-year gavage studies in F344/N rats and B6C3F_1 mice (Table 1; NTP, 1986). Benzene also caused treatment-related cancers in gavage (French *et al.*, 2001) and inhalation studies (Recio *et al.*, 2006) in $p53^{+/-}$ mice (Table 1). Benzene caused skin tumors in Tg.AC mice after dermal administration (Tennant *et al.*, 1995; Spalding *et al.*, 1999; Humble *et al.*, 2005).

Earlier benzene cancer studies in animals have been summarized by the International Programme on Chemical Safety, Environmental Health Criteria 150 (WHO, 1993). These studies also showed that benzene was carcinogenic in rodents after inhalation exposure (0 to 1,000 ppm) and oral gavage exposure (0 to 200 mg/kg) causing neoplasms in many target organs including the forestomach, harderian gland, liver, mammary gland, Zymbal's gland, and neoplasms within the hematopoietic and lymphoreticular systems (Snyder *et al.*, 1980, 1982, 1984; Goldstein *et al.*, 1982; Maltoni *et al.*, 1983, 1988; NTP, 1986; Cronkite *et al.*, 1989; Huff *et al.*, 1989; Farris *et al.*, 1993).

Microarray analyses of mouse bone marrow tissue before or after benzene exposure suggests that a critical event in toxicity is dysregulation of the p53 pathways resulting in alterations in cell cycle checkpoints, apoptosis, or the DNA repair system; these may be events leading to hematopoietic malignancies (Yoon *et al.*, 2003).

TABLE 1
Chemical-related Neoplasms in NTP Benzene Studies[a]

F344/N Male Rats (2-Year Gavage Study; NTP, 1986)

	0 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Zymbal's Gland				
Carcinoma	2/32 (6%)	6/46 (13%)	10/42 (24%)	17/42 (40%)
Oral Cavity				
Squamous Cell Papilloma or Carcinoma (Combined)	1/50 (2%)	9/50 (18%)	16/50 (32%)	19/50 (38%)
Skin				
Squamous Cell Papilloma	0/50 (0%)	2/50 (4%)	1/50 (2%)	5/50 (10%)
Squamous Cell Carcinoma	0/50 (0%)	5/50 (10%)	3/50 (6%)	8/50 (16%)

F344/N Female Rats (2-Year Gavage Study; NTP, 1986)

	0 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Zymbal's Gland				
Carcinoma	0/45 (0%)	5/40 (13%)	5/44 (11%)	14/46 (30%)
Oral Cavity				
Squamous Cell Papilloma or Carcinoma (Combined)	1/50 (2%)	5/50 (10%)	12/50 (24%)	9/50 (18%)

$B6C3F_1$ Male Mice (2-Year Gavage Study; NTP, 1986)

	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Zymbal's Gland				
Squamous Cell Carcinoma	0/43 (0%)	1/34 (3%)	4/40 (10%)	21/39 (54%)
Malignant Lymphoma	4/49 (8%)	9/48 (19%)	9/50 (18%)	15/49 (31%)
Lung				
Alveolar/bronchiolar Carcinoma	5/49 (10%)	11/48 (23%)	12/50 (24%)	14/49 (29%)
Alveolar/bronchiolar Adenoma or Carcinoma (Combined)	10/49 (20%)	16/48 (33%)	19/50 (38%)	21/49 (43%)
Harderian Gland				
Adenoma	0/49 (0%)	9/46 (20%)	13/49 (27%)	11/48 (23%)
Preputial Gland				
Squamous Cell Carcinoma	0/21 (0%)	3/28 (11%)	18/29 (62%)	28/35 (80%)
Forestomach				
Squamous Cell Papilloma or Carcinoma (Combined)	2/45 (4%)	2/42 (5%)	3/44 (7%)	5/38 (13%)

$B6C3F_1$ Female Mice (2-Year Gavage Study; NTP, 1986)

	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Zymbal's Gland				
Squamous Cell Carcinoma	0/43 (0%)	0/32 (0%)	1/37 (3%)	3/31 (10%)
Malignant Lymphoma	15/49 (31%)	24/45 (53%)	24/50 (48%)	20/49 (41%)
Liver				
Adenoma or Carcinoma (Combined)	4/49 (8%)	12/44 (27%)	13/50 (26%)	7/49 (14%)

TABLE 1
Chemical-related Neoplasms in NTP Benzene Studies

$B6C3F_1$ Female Mice (2-Year Gavage Study; NTP, 1986) (continued)

	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Lung				
Alveolar/bronchiolar Adenoma	4/49 (8%)	2/42 (5%)	5/50 (10%)	9/49 (18%)
Alveolar/bronchiolar Carcinoma	0/49 (0%)	3/42 (7%)	6/50 (12%)	6/49 (12%)
Alveolar/bronchiolar Adenoma or Carcinoma (Combined)	4/49 (8%)	5/42 (12%)	10/50 (20%)	13/49 (27%)
Harderian Gland				
Adenoma or Carcinoma (Combined)	5/48 (10%)	6/44 (14%)	10/50 (20%)	10/47 (21%)
Mammary Gland				
Carcinoma	0/49 (0%)	2/45 (4%)	5/50 (10%)	10/49 (20%)
Carcinosarcoma	0/49 (0%)	0/45 (0%)	1/50 (2%)	4/49 (8%)
Forestomach				
Squamous Cell Papilloma or Carcinoma (Combined)	1/42 (2%)	3/40 (8%)	6/45 (13%)	5/42 (12%)
Ovary				
Granulosa Cell Tumors	1/47 (2%)	1/44 (2%)	6/49 (12%)	7/48 (15%)
Benign Mixed Tumors	0/47 (0%)	1/44 (2%)	12/49 (24%)	7/48 (15%)

Male $p53^{+/-}$ Haploinsufficient Mice (26-Week Gavage Study; French *et al.*, 2001)

	0 mg/kg	100 mg/kg	200 mg/kg
Sarcoma	0/29 (0%)	7/20 (35%)	16/39 (41%)
Thymic Lymphoma	0/29 (0%)	1/20 (5%)	3/39 (8%)
Pancreas			
Acinar carcinoma	0/29 (0%)	0/29 (0%)	1/39 (3%)

Male $p53^{+/-}$ Mice (26-Week Inhalation Study; Recio *et al.*, 2006)

	0 ppm	100 ppm 3 days/week, 10 hours/day
Thymic Lymphoma	0/45 (0%)	4/43 (9%)

Male $p53^{+/-}$ Mice (1-Year Inhalation Study; Recio *et al.*, 2006)

	0 ppm	100 ppm 5 days/week, 6 hours/day	100 ppm 3 days/week, 10 hours/day	200 ppm 3 days/week, 5 hours/day
Thymic Lymphoma	1/45 (2%)	33/45 (73%)	37/43 (86%)	16/44 (36%)

Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice (27-Week Gavage Study; current study)

0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Lymphoma				
0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	5/15 (33%)

[a] All doses (except control) equal to 3,000 ppm × hours/week, exposure terminated at week 52, animals held up to 80 weeks.

Humans

Benzene is considered a known human carcinogen based on sufficient evidence in humans (IARC, 1982, 1987; NTP, 1986, 2004). Occupational studies provided much of the evidence of benzene's carcinogenicity in humans, where workers are generally exposed to benzene by inhalation and at much higher levels than the general public (USEPA, 1998). The evidence for the carcinogenic effects of benzene is supported by epidemiology studies, animal data, and mechanistic research. Studies of occupational inhalation exposure to benzene associate exposure with acute nonlymphocytic leukemia, preleukemia, and aplastic anemia. Using a linear dose-response curve, the USEPA (1998) estimated that the inhalation leukemia risk of 1 ppm of benzene was 7.1×10^{-3} to 2.5×10^{-2}. This risk estimate is based on benzene exposure in Pliofilm rubber workers at three plants in Ohio (Rinsky *et al.*, 1981, 1987).

The National Cancer Institute and the Chinese Academy of Preventive Medicine reported on cancer outcome in 74,828 benzene exposed workers from 1972 to 1987 at 672 factories in 12 Chinese cities (USEPA, 1998; Hayes *et al.*, 2001). While the workers in this study may have been exposed to chemicals other than benzene, this study provides additional data to show that benzene exposure is associated with hematotoxicity and leukemia.

Benzene effects on bone marrow cells with subsequent development of cancer may be due to the interactive effects of multiple genotoxic benzene metabolites, although epigenetic effects of benzene may also contribute to the cancer process (USEPA, 1998). Smith (1996) outlined proposed mechanisms in benzene-induced leukemia (Figure 2).

GENETIC TOXICITY

The genetic toxicity test data for benzene have been reviewed (Dean, 1978; NTP, 1986; IARC, 1987; Waters *et al.*, 1988; WHO, 1993), but no clear consensus on the mechanism of benzene-induced chromosomal damage and carcinogenesis has been reached (Eastmond, 2000; Whysner, 2000). Benzene is not active in bacterial gene

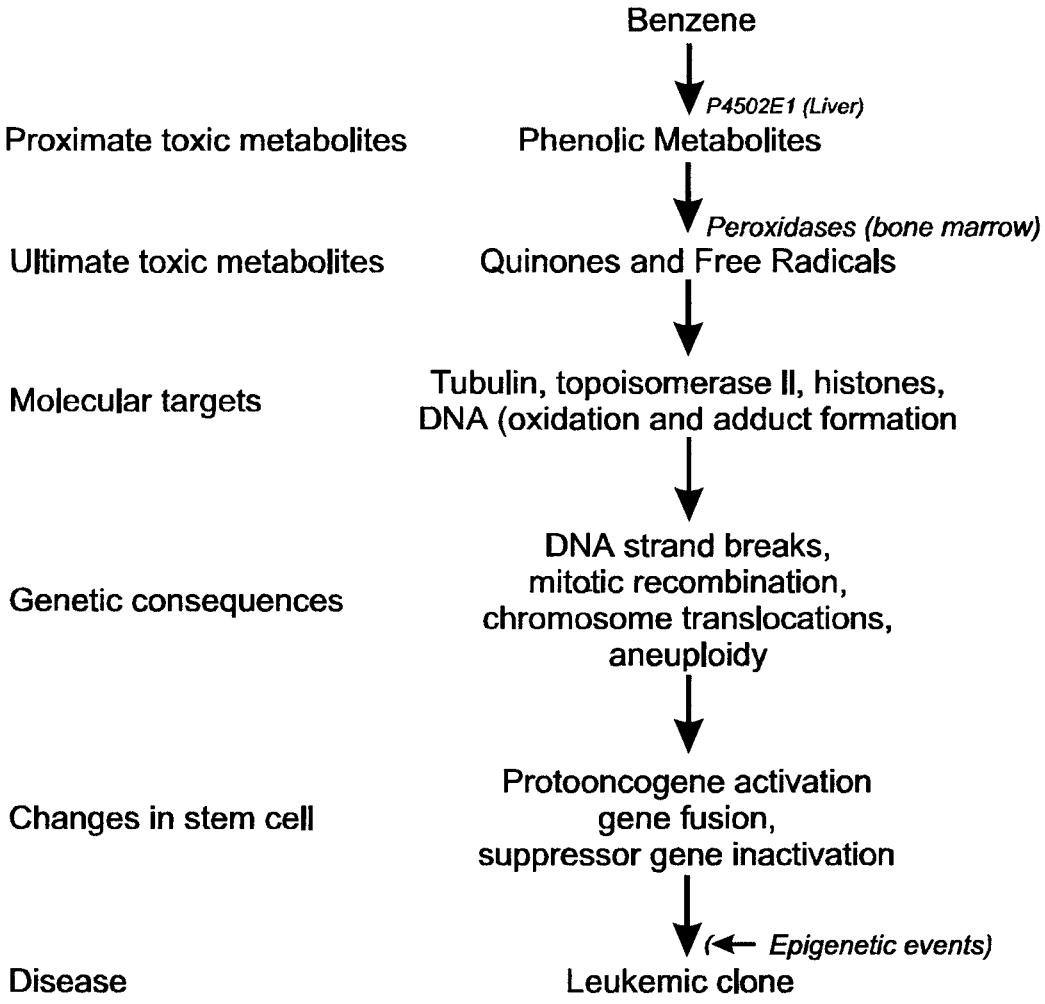


FIGURE 2
A Mechanistic Hypothesis of Benzene-Induced Leukemia (Smith, 1996)

mutation assays (Zeiger and Haworth, 1985), mammalian gene mutation assays using mouse lymphoma L5178Y/tk$^{+/-}$ cells (Myhr *et al.*, 1985), or germ cell mutation assays in male *Drosophila melanogaster* (Foureman *et al.*, 1994). However, under proper conditions of activation, it has been shown to induce mutations (Tsutsui *et al.*, 1997), DNA damage, sister chromatid exchanges, and aneuploidy in some *in vitro* mammalian cell systems (Gulati *et al.*, 1989; Tsutsui *et al.*, 1997). IARC (1987) also reported positive results in chromosomal aberration studies with benzene *in vitro*. Although some sporadic positive responses have been noted in mutagenicity tests *in vitro*, the mutagenicity of benzene is better studied *in vivo* because its metabolic pathways are complex and a variety of active metabolites are produced throughout its biotransformation. The literature describing benzene's ability to induce chromosomal damage *in vivo* in a variety of test systems is extensive. The earlier studies have been reviewed, as previously cited. More recent investigations have shown that benzene induces micronuclei in peripheral blood, bone marrow, and spleen cells of mice exposed via gavage (Choy *et al.*, 1985; MacGregor *et al.*, 1990; Chen *et al.*, 1994) and in lung cells of mice exposed to a single inhalation dose (Ranaldi *et al.*, 1998). Although bone marrow cell micronuclei in these studies originated predominantly from chromosome breakage, the majority of micronuclei seen in splenocytes, as indicated by fluorescent centromeric probes, arose from aneuploidy events (Chen *et al.*, 1994); micronuclei in lung cells resulted from both chromosomal breakage and aneuploidy events (Ranaldi *et al.*, 1998). Benzene also induced chromosomal aberrations in mouse bone marrow cells (Tice *et al.*, 1980) and lymphocytes (Rithidech *et al.*, 1987) following inhalation exposure, and increased chromosomal breakage was observed in differentiating spermatogonial cells of CD-1 mice given benzene as a single oral dose (Ciranni *et al.*, 1991). In humans, increases in numerical and structural chromosomal damage in lymphocytes of benzene-exposed workers have been well documented (Smith, 1996; Marcon *et al.*, 1999; Zhang *et al.*, 1999; and Kašuba *et al.*, 2000).

Background on Genetically Altered Mice

The CDKN2A genetic locus contains two important tumor suppressor genes located on chromosome 9, 4, and 5 in the human, mouse, and rat, respectively (NCBI, 2005). The locus is unique in that alternate splice variants produce

two different tumor suppressor proteins (Sherr and Weber, 2000; Sherr and McCormick, 2002; Lowe and Sherr, 2003). The $p16^{Ink4a}$ and $p19^{Arf}$ variants have exons 2 and 3 in common, but use different exon 1 (alpha and beta). Expression of these two splice variants is conserved across mammalian species. Mouse $p19^{Arf}$ and human $p14^{Arf}$ polypeptides are approximately 50% identical, and mouse $p16^{INK4}$ and human $p16^{INK4a}$ proteins are approximately 72% identical (Quelle *et al.*, 1995).

The two proteins translated from the mRNA expressed from CDKN2A are a p16-KDa protein and a p19 KDa protein (or p14 KDa protein in humans) (Serrano *et al.*, 1996). The p16 protein ($p16^{INK4a}$, inhibitor of kinase 4a) is a cell cycle regulatory protein that binds to cyclin dependent kinase 4 or 6 (CDK4/6) and inhibits the catalytic activity of the CDK/cyclin D complex and the phosphorylation of retinoblastoma protein. Since loss of the normal function of $p16^{INK4a}$ leads to uncontrolled cell growth, p16 is classified as a tumor suppressor gene (Serrano *et al.*, 1993). The second protein coded, $p19^{Arf}$ (Arf, alternative reading frame), induces G1 arrest and apoptosis. The 19Arf protein binds to MDM2 and neutralizes MDM2 inhibition of p53 (Sherr and Weber, 2000).

The targeted deletion of exons 2 and 3 of the Cdkn2a gene by a homologous recombination resulted in the elimination of both $p16^{INK4a}$ and $p19^{Arf}$ proteins (Serrano *et al.*, 1996). Homozygous null $Cdkn2a^{-/-}$ (or $Cdkn2a^{-/-}$) are viable and fertile (Serrano *et al.*, 1996). On inspection, these animals appear normal until about 2 months of age, but histological analysis of the spleen shows a mild proliferative expansion of the white pulp and the presence of numerous megakaryocytes and lymphoblasts in the red pulp. The $p16^{-/-}$ mice develop tumors at an average age of 29 weeks. Lymphomas and fibrosarcomas are two common types of tumors seen in this $Cdkn2a^{-/-}$. In contrast, the $Cdkn2a^{+/-}$ mouse does not usually develop any obvious tumors or display compromised health until after 36 weeks (Serrano *et al.*, 1996).

Deletions in the Cdkn2a gene predisposes both rodents and humans to cancer at multiple organ sites (Sharpless and DePinho, 1999). The complete loss of Cdkn2a gene(s) function is observed in approximately 10% of small cell

lung tumors, 30% of esophageal tumors, 55% of gliomas, 100% of pancreatic tumors, and 20% of head and neck tumors.

Transition from G1 to S phase in the mammalian cell cycle is under complex regulatory control, and one G1-S regulatory pathway involves $p16^{INK4a}$ protein. $P16^{INK4a}$ inhibits the cdk4/cyclin D1 complex, preventing cdk4 from phosphorylating pRb, and thus ensuring that pRb maintains G1 arrest. Disruption of this pathway, by $p16^{INK4a}$ gene mutations, perturbs the cell cycle (Serrano *et al.*, 1993) and in the case of these Cdkn2a genetically altered mice (Serrano *et al.*, 1993) results in more cell proliferation (Figure 3).

Serrano *et al.* (1996) report that treatment with DMBA and UV light causes an earlier onset of fibrosarcoma and lymphoma in the $p16^{-/-}$ mouse (at 8 to 10 weeks) and the $p16^{-/+}$ mouse (7 to 20 weeks).

STUDY RATIONALE

The purpose of this benzene study and the glycidol and phenolphthalein studies (NTP, 2006a,b) was to determine if a mouse with a deletion at the p16 gene locus (CDKN2), a locus that codes for two tumor suppressor genes, would enable the identification of carcinogenic chemicals in a shorter time frame and with fewer animals than the traditional 2-year NTP cancer study. These three chemicals were all multisite carcinogens in the NTP 2-year bioassays (NTP 1986, 1990, 1996). This study reports the findings from the benzene study.

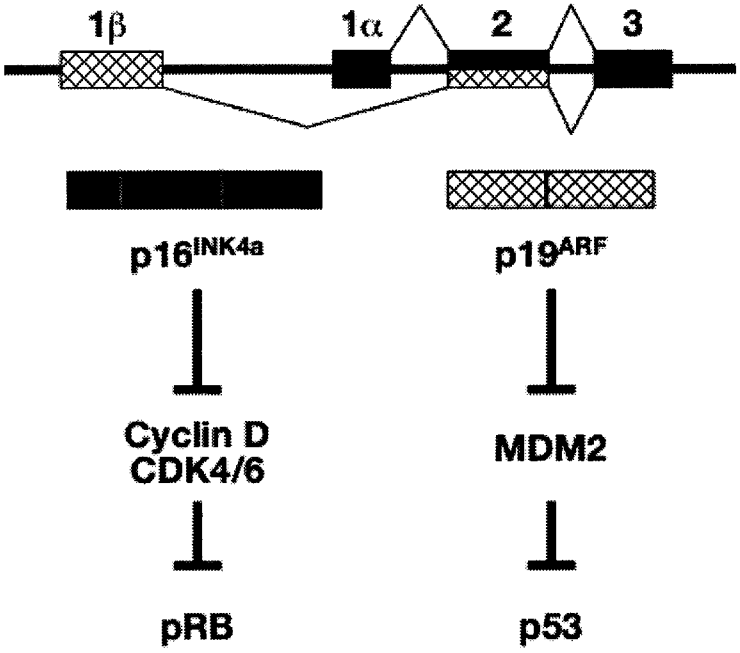


FIGURE 3
The *INK4a/ARF* Locus. The open reading frames $p16^{INK4a}$ (in black) and $p19^{ARF}$ (in crosshatch) are shown. Each has a unique first exon that then splices to a common second exon, but in alternate reading frames. $P16^{INK4a}$ inhibits cdk4/6 activity producing retinoblastoma phosphorylation, which induces cell cycle arrest. $P19^{ARF}$ inhibits MDM2-mediated degradation of p53 (Sharpless, 2005).

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF BENZENE

Benzene was obtained from Sigma Aldrich Chemical Co. (Milwaukee, WI) in one lot (00358CS). Identity and purity analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (RTI) (Research Triangle Park, NC) and the study laboratory, Battelle Columbus Operations (Columbus, OH) (Appendix D). Reports on analyses performed in support of the benzene study are on file at the National Institute of Environmental Health Sciences.

Lot 00358CS, a colorless liquid, was identified as benzene by RTI using infrared spectroscopy and proton nuclear magnetic resonance (NMR) spectroscopy and by the study laboratory using infrared spectroscopy. Spectra were in agreement with the structure of benzene, literature spectra (*Aldrich*, 1981, 1983, 1985), and spectra from a frozen reference standard of the same lot.

The purity of lot 00358CS was determined by RTI and by the study laboratory using gas chromatography (GC) by flame ionization. The purity profile performed by RTI detected one major peak and one impurity peak with an area that was less than 0.1% of the total peak area. The overall purity of lot 00358CS was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored at room temperature (25° C) in amber glass bottles with Teflon®-lined lids, protected from light. Stability was monitored twice during the 27-week study with GC by flame ionization; no degradation of the bulk chemical was detected. The purity profile performed by the study laboratory detected one major peak with a purity of 99.3% relative to a frozen reference standard of the same lot.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing benzene with corn oil (Spectrum Chemicals and Laboratory Products, Inc., Gardena, CA) to give the required concentrations. Because animal room sample analyses indicated probable evaporative loss of benzene from the dose formulations during administration, dose formulations prepared on January 4, 2000, or later were prepared with 5% less corn oil than specified in the original SOP for dose formulation. The dose formulations were stored at room temperature (25° C) in amber glass bottles capped with Teflon®-lined lids for up to 35 days.

Stability studies of a 1.0 mg/mL dose formulation of benzene in corn oil were performed by RTI with GC by flame ionization. Stability was confirmed for up to 35 days for dose formulations stored in the dark in sealed glass bottles at refrigerator temperature (5° C) and for up to 3 hours exposed to air and light under simulated dosing conditions.

Periodic analyses of the dose formulations of benzene were conducted by the study laboratory using GC by flame ionization. During the 27-week study, the dose formulations were analyzed four times and postadministration animal room samples were analyzed once (Table D3). All 12 of the dose formulations analyzed and used in the study were within 10% of the target concentrations; all of the four analyzed animal room samples of the dose formulations were within 15% of the target concentration. Periodic analyses of the corn oil vehicle by the study laboratory demonstrated peroxide concentrations below the acceptable limit of 3 mEq/kg.

27-Week Study

Study Design

Groups of 15 male and 15 female mice were administered benzene in corn oil by gavage at doses of 0, 25, 50, 100, or 200 mg benzene/kg body weight 5 days per week for 27 to 28 weeks. The doses selected for use in the 27-week

study were chosen to overlap those used for $p53^{+/-}$ mice in the French *et al.* (2001) gavage study and for $B6C3F_1$ mice in the NTP (1986) 2-year gavage study.

Source and Specification of Animals

Male and female heterozygous B6.129-$Cdkn2a^{tm1Rdp}$ N3 (i.e., haploinsufficient $p16^{Ink4a}/p19^{Arf}$) mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY) for use in the 27-week study. The N1 male mice homozygous null for the Cdkn2a deletion were backcrossed to inbred C57BL/6 females from Taconic Laboratory to produce male and female B6.129-$Cdkn2^{atm1Rdp}$ haploinsufficient or $p16^{Inka}/p19^{Arf+/-}$ mice (Serrano *et al.*, 1996). The genetic background of these mice was: 80% C57BL/6, 19% 129/Sv, and 1% SJL. This line, designated 5003 by Taconic Laboratory, was embryo cryopreserved in 2003. Upon receipt, the mice were 3 to 4 weeks old. Animals were quarantined for 22 days and were 6 to 7 weeks old on the first day of the study. Before the study began, five male and five female mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Blood samples were collected from up to five male and five female sentinel animals at 4 weeks and at study termination. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b). All results were negative.

Mice were housed individually. Feed and water were available *ad libitum*. Cages and racks were rotated weekly. Further details of animal maintenance are given in Table 2.

Clinical Examinations and Pathology

All mice were observed twice daily. Clinical findings were recorded weekly, to coincide with body weight collection, and at the end of the study. The mice were weighed initially, weekly, and at the end of the study.

Blood for hematology analysis was collected from the retroorbital sinus of mice under carbon dioxide anesthesia at 13 and 27 weeks. Samples were placed in microcollection tubes (Sarstedt, Inc., Nümbrecht, Germany) coated with potassium EDTA. Hematocrit; erythrocyte, platelet, and leukocyte counts; mean cell hemoglobin; and mean cell

hemoglobin concentration were determined with a Cell-Dyn® hematology analyzer (Abbott Laboratories, Abbott Park, IL). Differential leukocyte counts were determined microscopically from smears stained with a Wright-Giemsa stain. A Miller Disc was used to determine reticulocyte counts from smears prepared with blood stained with new methylene blue.

Complete necropsies and microscopic examinations were performed on all mice. The heart, right kidney, liver, lung, right testis, and thymus were weighed. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin for microscopic examination. Histopathologic examinations were performed on all mice. Table 2 lists the tissues and organs routinely examined.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy; the slide and tissue counts were verified, and the histotechnique was evaluated. For the 27-week study, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the bone marrow, lymph nodes, skin, spleen, and thymus.

The quality assessment report and the reviewed slides were submitted to the NTP Pathology Working Group (PWG) chairperson, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologists, or lesions of general interest were presented by the PWG chairperson to the NTP pathologist. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist,

reviewing pathologist(s), the PWG chairperson, and the NTP pathologist. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al.* (1986).

TABLE 2
Experimental Design and Materials and Methods in the 27-Week Gavage Study of Benzene

Study Laboratory	Battelle Columbus Operations (Columbus, OH)
Strain and Species	Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice (Heterozygous B6.129-$Cdkn2a^{tm1Rdp}$ N3)
Animal Source	Taconic Laboratory Animals and Services (Germantown, NY)
Time Held Before Study	22 days
Average Age When Studies Began	6 to 7 weeks
Date of First Dose	October 29, 1999
Duration of Dosing	5 days/week for 27 weeks
Date of Last Dose	May 2-4, 2000
Necropsy Dates	May 3-5, 2000
Average Age at Necropsy	33 to 34 weeks
Size of Study Groups	15 males and 15 females
Method of Distribution	Animals were distributed randomly into groups of approximately equal initial mean body weights.
Animals per Cage	1
Method of Animal Identification	Tail tattoo and ear tag
Diet	Irradiated NTP-2000 pelleted feed (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*

TABLE 2
Experimental Design and Materials and Methods in the 27-Week Gavage Study of Benzene

Water
Tap water (City of Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*

Cages
Polycarbonate (Lab Products, Inc., Maywood, NJ), changed weekly

Racks
Stainless steel (Lab Products, Inc., Maywood, NJ), changed every 2 weeks

Bedding
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corp., Montville, NJ), changed weekly

Cage Filters
DuPont 2024 spun-bonded polyester (Snow Filtration Co., Cincinnati, OH), changed every 2 weeks

Animal Room Environment
Temperature: 72° ± 3° F
Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day
Room air changes: 10/hour

Doses
0, 25, 50, 100, or 200 mg/kg in corn oil (dosing volume 10 mL/kg body weight)

Type and Frequency of Observation
Observed twice daily; animals were weighed initially, weekly, and at the end of the study; clinical findings were recorded weekly.

Method of Sacrifice
CO_2 asphyxiation

Necropsy
Necropsies were performed on all animals. Organs weighed were heart, right kidney, liver, lungs, right testis, and thymus.

Clinical Pathology
Blood was collected via the retroorbital sinus from all animals during weeks 13 and 27 for hematology.
Hematology: hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials

Histopathology
Histopathology was performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow (femur and sternum), brain, clitoral gland, large intestine (colon, cecum, rectum), small intestine (duodenum, ileum, jejunum), heart, kidney, liver, lung, lymph nodes (mandibular, mesenteric), mammary gland, ovary, pituitary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis, thymus, thyroid gland, urinary bladder, and uterus.

STATISTICAL METHODS

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). Animals found dead of other than natural causes or missing were censored; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A2, A3, and A4 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test (Gart *et al.*, 1979) and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the overall proportion of affected animals, were used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and

implausible values were eliminated from the analysis. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973).

QUALITY ASSURANCE METHODS

The 27-week study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records were submitted to the NTP Archives, this study was audited retrospectively by an independent quality assurance contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Report.

GENETIC TOXICOLOGY

Mouse Peripheral Blood Micronucleus Test Protocol

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At 6.5, 13, 19.5, and 27 weeks, peripheral blood samples were obtained from male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each of up to 15 animals per dose group. In addition, the percentage of polychromatic erythrocytes among 1,000 total erythrocytes was determined for each animal as a measure of benzene-induced bone marrow toxicity.

The results were tabulated as the mean of the pooled results from all animals within a treatment group, plus or minus the standard error of the mean. The frequency of micronucleated cells among NCEs was analyzed by a statistical software package that tested for increasing trend over dose groups using a one-tailed Cochran-Armitage

trend test, followed by pairwise comparisons between each dosed group and the vehicle control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dosed group is less than or equal to 0.025 divided by the number of dosed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects. Because these studies were not repeated, the results of the micronucleus trials were accepted without replication.

RESULTS

MICE

27-WEEK STUDY

Survival

Estimates of 27-week survival probabilities for male and female mice are shown in Table 3. Survival of all dosed groups of male and female mice was similar to that of the vehicle controls; all animals survived until the end of the study except one male administered 200 mg/kg.

TABLE 3
Survival of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

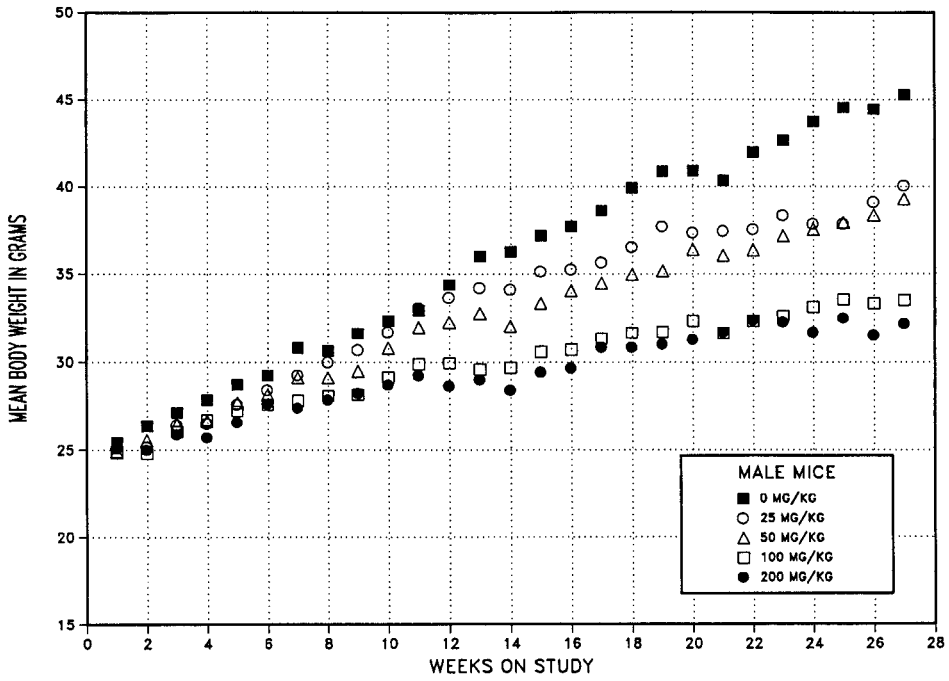
	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Animals initially in study	15	15	15	15	15
Moribund	0	0	0	0	1
Animals surviving to study termination	15	15	15	15	14
Percent probability of survival at end of study[a]	100	100	100	100	93
Mean survival (days)[b]	188	188	188	188	176
Survival analysis[c]	P=0.289	—[d]	—	—	P=1.000
Female					
Animals initially in study	15	15	15	15	15
Animals surviving to study termination	15	15	15	15	15
Percent probability of survival at end of study	100	100	100	100	100
Mean survival (days)	189	189	189	189	189
Survival analysis	—	—	—	—	—

[a] Kaplan-Meier determinations
[b] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[c] The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns.
[d] Value of statistic cannot be computed.

Body Weights, Organ Weights, and Clinical Findings

Mean body weights of males administered 50 mg/kg or greater were generally less than those of the vehicle controls throughout the study, and those of 25 mg/kg males were less after week 13 (Figure 4 and Tables 4 and 5). By week 13, mean body weights of males in the 25, 50, 100, and 200 mg/kg groups were 5%, 9%, 18%, and 19% less than that of the vehicle controls, respectively. Mean body weights of 200 mg/kg females were less than those of the vehicle controls after week 17. Treatment-related clinical findings in 25 mg/kg or greater males and 50 mg/kg or greater females included black, brown, or gray discoloration (pigmentation) of the feet. Male mice in the 100 and 200 mg/kg groups also had dark pigmentation of the nose.

Compared to the vehicle controls, the absolute liver and thymus weights of all dosed groups of males and the absolute right testis weights of 50 mg/kg or greater males were significantly decreased (Table C1). The relative thymus weights of 50 mg/kg or greater males were significantly decreased.



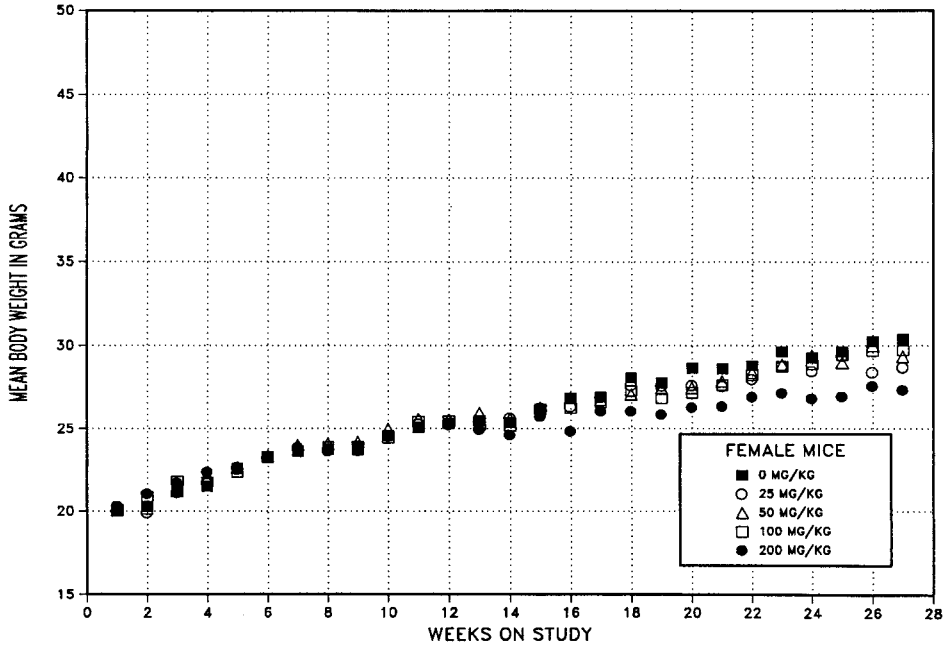


FIGURE 4
Growth Curves for Male and Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Administered Benzene by Gavage for 27 Weeks

TABLE 4
Mean Body Weights and Survival of Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

Weeks on Study	Vehicle Control		25 mg/kg			50 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	25.4	15	25.1	99	15	24.9	98	15
2	26.4	15	25.2	96	15	25.5	97	15
3	27.1	15	26.4	97	15	26.5	98	15
4	27.8	15	26.5	95	15	26.6	96	15
5	28.7	15	27.6	96	15	27.7	97	15
6	29.2	15	28.4	97	15	28.2	97	15
7	30.8	15	29.3	95	15	29.1	95	15
8	30.6	15	30.0	98	15	29.1	95	15
9	31.6	15	30.7	97	15	29.5	93	15
10	32.3	15	31.7	98	15	30.8	95	15
11	32.9	15	33.0	100	15	31.9	97	15
12	34.4	15	33.6	98	15	32.2	94	15
13	36.0	15	34.2	95	15	32.7	91	15
14	36.3	15	34.1	94	15	32.0	88	15
15	37.2	15	35.1	94	15	33.3	90	15
16	37.7	15	35.2	93	15	34.0	90	15
17	38.6	15	35.6	92	15	34.5	89	15
18	39.9	15	36.5	92	15	35.0	88	15
19	40.9	15	37.7	92	15	35.2	86	15
20	40.9	15	37.4	91	15	36.4	89	15
21	40.3	15	37.4	93	15	36.1	90	15
22	41.9	15	37.5	90	15	36.4	87	15
23	42.6	15	38.3	90	15	37.2	87	15
24	43.7	15	37.9	87	15	37.5	86	15
25	44.5	15	37.9	85	15	37.9	85	15
26	44.4	15	39.1	88	15	38.4	87	15
27	45.3	15	40.0	88	15	39.3	87	15
Mean for weeks								
1-13	30.2		29.4	97		28.8	97	
14-27	41.0		37.1	91		35.9	88	

TABLE 4
Mean Body Weights and Survival of Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

Weeks on Study	100 mg/kg Av. Wt. (g)	100 mg/kg Wt. (% of controls)	100 mg/kg No. of Survivors	200 mg/kg Av. Wt. (g)	200 mg/kg Wt. (% of controls)	200 mg/kg No. of Survivors
1	24.8	98	15	25.1	99	15
2	24.8	94	15	25.0	95	15
3	26.0	96	15	25.9	96	14
4	26.7	96	15	25.7	92	14
5	27.2	95	15	26.6	93	14
6	27.6	95	15	27.6	95	14
7	27.8	90	15	27.4	89	14
8	28.1	92	15	27.8	91	14
9	28.2	89	15	28.2	89	14
10	29.1	90	15	28.7	89	14
11	29.9	91	15	29.2	89	14
12	29.9	87	15	28.6	83	14
13	29.6	82	15	29.0	81	14
14	29.7	82	15	28.4	78	14
15	30.6	82	15	29.4	79	14
16	30.7	81	15	29.7	79	14
17	31.3	81	15	30.8	80	14
18	31.6	79	15	30.8	77	14
19	31.7	78	15	31.0	76	14
20	32.3	79	15	31.3	77	14
21	31.6	78	15	31.6	78	14
22	32.3	77	15	32.3	77	14
23	32.6	77	15	32.3	76	14
24	33.1	76	15	31.7	73	14
25	33.5	75	15	32.5	73	14
26	33.3	75	15	31.5	71	14
27	33.5	74	15	32.1	71	14
Mean for weeks						
1-13	27.7	92		27.3	91	
14-27	32.0	78		31.1	76	

TABLE 5
Mean Body Weights and Survival of Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

Weeks on Study	Vehicle Control		25 mg/kg			50 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	20.0	15	20.0	100	15	20.0	100	15
2	20.3	15	19.9	98	15	20.1	99	15
3	21.2	15	21.1	100	15	21.2	100	15
4	21.5	15	21.7	101	15	22.1	103	15
5	22.6	15	22.6	100	15	22.6	100	15
6	23.2	15	23.2	100	15	23.3	100	15
7	23.6	15	23.8	101	15	24.0	102	15
8	23.7	15	23.6	100	15	24.1	102	15
9	23.7	15	23.6	100	15	24.2	102	15
10	24.6	15	24.6	100	15	25.0	102	15
11	25.1	15	25.3	101	15	25.6	102	15
12	25.3	15	25.2	100	15	25.5	101	15
13	25.5	15	25.4	100	15	25.9	102	15
14	25.4	15	25.6	101	15	25.6	101	15
15	26.2	15	26.2	100	15	26.3	100	15
16	26.8	15	26.3	98	15	26.8	100	15
17	26.9	15	26.9	100	15	26.7	99	15
18	28.1	15	27.6	98	15	27.0	96	15
19	27.8	15	27.5	99	15	27.4	99	15
20	28.6	15	27.6	97	15	27.4	96	15
21	28.6	15	27.5	96	15	27.8	97	15
22	28.8	15	27.9	97	15	28.3	98	15
23	29.6	15	28.7	97	15	28.8	97	15
24	29.3	15	28.4	97	15	29.4	100	15
25	29.6	15	29.4	99	15	28.9	98	15
26	30.3	15	28.4	94	15	30.0	99	15
27	30.4	15	28.7	94	15	29.3	96	15
Mean for weeks								
1-13	23.1		23.1	100		23.4	101	
14-27	28.3		27.6	98		27.8	98	

TABLE 5
Mean Body Weights and Survival of Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

Weeks on Study	100 mg/kg Av. Wt. (g)	100 mg/kg Wt. (% of controls)	100 mg/kg No. of Survivors	200 mg/kg Av. Wt. (g)	200 mg/kg Wt. (% of controls)	200 mg/kg No. of Survivors
1	20.1	101	15	20.3	102	15
2	20.9	103	15	21.0	103	15
3	21.8	103	15	21.7	102	15
4	21.7	101	15	22.4	104	15
5	22.4	99	15	22.5	100	15
6	23.3	100	15	23.2	100	15
7	23.6	100	15	23.8	101	15
8	23.9	101	15	23.7	100	15
9	23.9	101	15	23.9	101	15
10	24.4	99	15	24.5	100	15
11	25.4	101	15	25.1	100	15
12	25.5	101	15	25.3	100	15
13	25.2	99	15	24.9	98	15
14	25.1	99	15	24.6	97	15
15	26.1	100	15	25.7	98	15
16	26.2	98	15	24.8	93	15
17	26.5	99	15	26.0	97	15
18	27.3	97	15	26.0	93	15
19	26.8	96	15	25.9	93	15
20	27.2	95	15	26.3	92	15
21	27.6	97	15	26.3	92	15
22	28.2	98	15	26.9	93	15
23	28.7	97	15	27.1	92	15
24	28.9	99	15	26.8	92	15
25	29.4	99	15	26.9	91	15
26	29.7	98	15	27.5	91	15
27	29.7	98	15	27.3	90	15
Mean for weeks						
1-13	23.2	101		23.3	101	
14-27	27.7	98		26.3	93	

Hematology

At weeks 13 and 27, a dose-related decrease in the erythron occurred in males and females. The erythron decrease was shown by decreases in the hematocrit, hemoglobin, and erythrocyte count values in all dosed males and in the 100 mg/kg or greater females (Tables 6 and B1). Male mice were more severely affected with a greater than 20% erythron decrease at 200 mg/kg compared to the 10% or less decrease in 200 mg/kg females. Also, at 13 and 27 weeks, the erythron decrease was accompanied by an increase in erythrocyte size, which was shown by the dose-related increase in mean cell volumes. The males were more affected than the females with an approximately 15% increase compared to approximately 4% at 200 mg/kg, respectively. Another consistent finding was a dose-related leukopenia, evidenced by decreases in the leukocyte counts. Males were affected at a lower dose and to a greater magnitude than females. For example, for 200 mg/kg males, leukocyte counts were approximately 20% of the control counts while those in 200 mg/kg females were approximately 68% of the control counts. The decreases in leukocyte counts were primarily related to decreases in the lymphocyte counts. Segmented neutrophil counts were also decreased in the 100 and 200 mg/kg groups at week 27. These findings were consistent with the predictable hematopoietic effects of benzene that can result in anemia, leukopenia, thrombocytopenia, or some combination of these.

TABLE 6
Selected Hematology Data for Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
n					
Week 13	15	15	15	15	14
Week 27	15	15	15	15	13
Hematocrit (%)					
Week 13	51.5 ± 0.5	50.2 ± 0.7	47.3 ± 0.6**	44.3 ± 0.6**	40.9 ± 0.9**
Week 27	48.4 ± 0.4	47.1 ± 0.9	44.8 ± 0.5**	43.5 ± 0.7**	36.4 ± 1.3**
Hemoglobin (g/dL)					
Week 13	17.1 ± 0.1	16.4 ± 0.2**	15.4 ± 0.2**	14.6 ± 0.3**	13.4 ± 0.3**
Week 27	16.3 ± 0.2	15.8 ± 0.3	15.2 ± 0.2**	14.4 ± 0.2**	12.1 ± 0.4**
Erythrocytes (10^6/μL)					
Week 13	11.51 ± 0.11	10.74 ± 0.16**	10.04 ± 0.15**	9.14 ± 0.14**	8.31 ± 0.22**
Week 27	10.92 ± 0.12	10.20 ± 0.20*	9.55 ± 0.12**	8.99 ± 0.16**	7.11 ± 0.27**
Mean cell volume (fL)					
Week 13	44.8 ± 0.2	46.7 ± 0.2**	47.1 ± 0.2**	48.6 ± 0.2**	49.4 ± 0.3**
Week 27	44.3 ± 0.3	46.2 ± 0.2**	46.9 ± 0.2**	48.4 ± 0.3**	51.4 ± 0.7**
Leukocytes (10^3/μL)					
Week 13	4.20 ± 0.42	2.79 ± 0.24*	2.38 ± 0.15**	1.42 ± 0.07**	0.93 ± 0.08**
Week 27	5.25 ± 0.41	3.19 ± 0.27**	2.21 ± 0.18**	1.48 ± 0.15**	0.95 ± 0.06**
Segmented neutrophils (10^3/μL)					
Week 13	0.45 ± 0.05	0.41 ± 0.05	0.43 ± 0.05	0.41 ± 0.03	0.33 ± 0.03
Week 27	1.10 ± 0.14	0.81 ± 0.09	0.81 ± 0.12*	0.60 ± 0.11**	0.52 ± 0.04**
Lymphocytes (10^3/μL)					
Week 13	3.70 ± 0.39	2.33 ± 0.19*	1.93 ± 0.12**	0.99 ± 0.06**	0.59 ± 0.07**
Week 27	4.12 ± 0.43	2.35 ± 0.23**	1.38 ± 0.08**	0.87 ± 0.09**	0.42 ± 0.03**
Female					
n					
Week 13	15	15	15	14	15
Week 27	15	15	15	14	14
Hematocrit (%)					
Week 13	50.1 ± 0.5	50.2 ± 0.6	48.8 ± 0.6	47.4 ± 1.1*	46.7 ± 0.8**
Week 27	45.5 ± 0.5	45.6 ± 0.8	46.0 ± 0.8	44.3 ± 0.8	42.9 ± 0.5**
Hemoglobin (g/dL)					
Week 13	17.0 ± 0.2	16.7 ± 0.2	16.4 ± 0.2	15.8 ± 0.4**	15.7 ± 0.3**
Week 27	15.5 ± 0.2	15.4 ± 0.3	15.5 ± 0.3	14.9 ± 0.3	14.2 ± 0.2**
Erythrocytes (10^6/μL)					
Week 13	10.94 ± 0.12	10.83 ± 0.13	10.52 ± 0.13*	9.92 ± 0.26**	9.80 ± 0.17**
Week 27	10.02 ± 0.11	10.02 ± 0.21	10.04 ± 0.20	9.41 ± 0.17**	9.03 ± 0.11**
Mean cell volume (fL)					
Week 13	45.8 ± 0.2	46.4 ± 0.2*	46.4 ± 0.1*	47.9 ± 0.2**	47.7 ± 0.2**
Week 27	45.4 ± 0.2	45.6 ± 0.3	45.9 ± 0.2	47.1 ± 0.2**	47.5 ± 0.2**
Leukocytes (10^3/μL)					
Week 13	5.07 ± 0.50	4.84 ± 0.35	3.59 ± 0.22*	3.28 ± 0.16**	3.83 ± 0.31*
Week 27	6.25 ± 0.36	6.17 ± 0.48	4.71 ± 0.29**	3.41 ± 0.24**	3.74 ± 0.34**
Lymphocytes (10^3/μL)					
Week 13	4.53 ± 0.45	4.35 ± 0.33	3.13 ± 0.19*	2.77 ± 0.15**	3.03 ± 0.26**
Week 27	5.48 ± 0.32	5.03 ± 0.37	3.56 ± 0.18**	2.57 ± 0.21**	2.69 ± 0.22**

* Significantly different ($P \le 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \le 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of malignant lymphoma and nonneoplastic lesions in the bone marrow, spleen, thymus, lymph nodes, skin, and mammary gland. Summaries of the incidences of neoplasms and nonneoplastic lesions are presented in Tables A1, A2, A3, and A4.

Malignant Lymphoma: The incidence of malignant lymphoma was significantly increased in 200 mg/kg males compared to the vehicle controls, and the incidence exceeded the historical control range in male mice from the current 27-week benzene and phenolphthalein studies and 40-week aspartame and glycidol studies (Tables 7, A1, and E1). Neoplastic cells infiltrated multiple organs including the spleen, thymus, lymph node, kidney, lung, and/or brain. No malignant lymphomas occurred in females.

TABLE 7
Incidences of Malignant Lymphoma in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Overall Rate[a,b]	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	5/15 (33%)
Adjusted Rate[c]	0.0%	0.0%	0.0%	0.0%	33.3%
Terminal Rate[d]	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	4/14 (29%)
First Incidence (days)	—[f]	—	—	—	11
P value[e]	P<0.001	—[g]	—	—	P=0.021

[a] Historical incidence for the 27-week gavage studies with haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse vehicle control groups: 27 week: 1/30 (3%), range 0%-7%; 40 week: 2/30 (7%), range 0%-13%
[c] Number of animals with neoplasm per number of animals necropsied
[d] Observed incidence at terminal kill
[e] The result of the Cochran-Armitage trend test (Armitage, 1971) is in the vehicle control column and the results of the Fisher exact pairwise comparisons (Gart *et al.*, 1979) with the vehicle controls are in the dosed group columns.
[f] Not applicable; no neoplasms in animal group
[g] Value of statistic cannot be computed

Bone Marrow: Significantly increased incidences of minimal to mild atrophy occurred in the 100 and 200 mg/kg males compared to the vehicle controls (Tables 8 and A2). Atrophy was characterized by histologically detectable decreased cellularity of femoral and/or sternal bone marrow with increased amounts of adipocytes and/or blood vessels. Dose-related increased incidences and severities of hemosiderin pigmentation occurred in males. There were no benzene-related effects on bone marrow in female mice.

Spleen: Dose-related incidences of lymphoid follicle atrophy were significantly increased in 100 and 200 mg/kg male mice (Tables 8 and A2). Lymphoid follicle atrophy occurred in a few females in the 25, 50, and 200 mg/kg groups (Tables 8 and A4). Lymphoid follicle atrophy was characterized by decreased lymphoid follicle size, decreased variety of cell types, decreased numbers of lymphocytes, and sometimes a decreased overall size of splenic profile. Affected spleens tended to have smaller amounts of lymphoid tissue than the vehicle control mice, as well as having primarily small lymphocytes with no germinal centers and fewer intermingled histiocytes.

The incidence of hematopoietic cell proliferation was significantly increased in 200 mg/kg males compared to vehicle controls, and hematopoietic cell proliferation occurred in all groups of females (Tables 8 and A2). Hematopoietic cell proliferation consisted of increased blood precursor cells in the red pulp that generally caused some enlargement of the spleen; however, in the 200 mg/kg males, the enlargement did not offset the lymphoid atrophy.

Thymus: The incidences of atrophy in the 100 and 200 mg/kg males were significantly greater than that in the vehicle controls (Tables 8 and A2). Atrophy was characterized by decreased size of the thymic profile, decreased numbers of lymphocytes, and loss of corticomedullary distinction.

Lymph Nodes (Mandibular, Mediastinal, and Mesenteric): Significantly increased incidences of atrophy (mandibular, mediastinal, and mesenteric) occurred in 100 and 200 mg/kg males (Tables 8 and A2). The incidences of atrophy of the mesenteric lymph node were significantly increased in 100 and 200 mg/kg females,

TABLE 8
Incidences of Selected Nonneoplastic Lesions in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Bone Marrow[a]	15	15	15	15	15
Atrophy[b]	0	0	0	10** (1.1)[c]	12** (1.3)
Pigmentation Hemosiderin	1 (1.0)	13** (1.0)	13** (1.1)	15** (2.0)	14** (2.9)
Spleen	15	15	15	15	15
Hematopoietic Cell Proliferation	0	1 (1.0)	0	2 (1.0)	8** (1.3)
Lymphoid Follicle Atrophy	0	0	0	15** (1.8)	14** (2.9)
Thymus	15	15	15	15	15
Atrophy	0	0	0	7** (3.0)	13** (2.9)
Lymph Node (Mandibular)	15	15	15	14	14
Atrophy	0	0	0	6** (1.3)	11** (1.2)
Lymph Node (Mediastinal)	11	11	12	9	7
Atrophy	0	0	2 (1.0)	6** (1.3)	6** (1.7)
Lymph Node (Mesenteric)	15	15	14	15	15
Atrophy	1 (1.0)	2 (2.0)	2 (1.0)	13** (2.0)	13** (1.7)
Skin	15	15	15	15	15
Pigmentation	0	15** (1.1)	15** (1.6)	15** (2.3)	14** (2.6)
Female					
Spleen	15	15	15	15	15
Hematopoietic Cell Proliferation	5 (1.0)	5 (1.2)	2 (1.0)	3 (1.3)	7 (1.1)
Lymphoid Follicle Atrophy	0	1 (1.0)	1 (2.0)	0	1 (2.0)
Lymph Node (Mandibular)	14	15	15	15	15
Atrophy	0	1 (1.0)	2 (1.0)	4 (1.0)	0
Lymph Node (Mediastinal)	13	11	11	12	13
Atrophy	0	0	2 (1.5)	4* (1.3)	2 (1.0)
Lymph Node (Mesenteric)	15	15	15	15	15
Atrophy	0	2 (2.0)	3 (1.0)	8** (1.8)	6** (1.3)
Skin	15	15	15	15	15
Pigmentation	0	1 (1.0)	8** (1.0)	15** (1.1)	15** (1.1)
Mammary Gland	15	15	15	15	15
Hyperplasia	0	0	0	0	2 (1.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

and the incidence of atrophy of the mediastinal lymph node was significantly increased in 100 mg/kg females (Tables 8 and A4). Atrophy was characterized by decreased numbers of lymphocytes in the lymph nodes, decreased variety of cell-types in lymphatic nodules, increased sinusoidal spaces, and decreased medullary cords or germinal centers.

Skin: The incidences of skin pigmentation were significantly increased in all dosed groups of males and in 50 mg/kg or greater females, and the severity generally increased with increasing dose (Tables 8, A2, and A4). The males were more sensitive to this effect. The increased pigmentation (consistent with melanin) was detected in the epidermis of dosed mice from the paw but not the inguinal area. The pigmentation consisted of five or more separate foci per section of skin (minimal) to confluent regions over more than 1 mm (mild) to involvement of the entire epidermis (moderate).

Mammary Gland: Hyperplasia occurred in two female mice treated with 200 mg/kg benzene, but not in any other dosed groups (Tables 8 and A4).

GENETIC TOXICOLOGY

The frequency of micronucleated normochromatic erythrocytes (NCEs) was assessed in male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice at 6.5, 13, 19.5, and 26 weeks of exposure to benzene. At all four time points, a significant increase in micronucleated NCEs was observed in both sexes; the magnitude of the response increased with increasing duration of treatment (Table 9). At the 6.5-week sampling time, the response seen in female mice was weak, and no individual doses were statistically elevated over the control value. The trend test was positive, however, ($P<0.001$) and the result of the micronucleus test in female mice at 6.5 weeks was concluded to be positive. The male mice sampled at 6.5 weeks showed a highly significant increase in micronucleated NCEs at all four dose levels. At all subsequent sampling times, the frequency of micronucleated NCEs in male and female mice was significantly increased over the control at all four dose levels. Percent

TABLE 9
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Following Treatment with Benzene by Gavage for up to 27 Weeks[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]	PCEs[b] (%)
Male					
6.5 Weeks					
Corn Oil[d]		15	0.73 ± 0.18		2.460 ± 0.11
Benzene	25	15	1.87 ± 0.29	0.0001	2.507 ± 0.15
	50	15	4.87 ± 0.36	<0.0001	2.707 ± 0.13
	100	15	7.23 ± 0.49	<0.0001	2.607 ± 0.21
	200	14	7.64 ± 0.36	<0.0001	2.564 ± 0.17
			P=0.000[e]		
13 Weeks					
Corn Oil		15	1.03 ± 0.18		2.413 ± 0.10
Benzene	25	15	2.83 ± 0.32	<0.0001	2.413 ± 0.11
	50	15	2.03 ± 0.29	0.0032	1.853 ± 0.11
	100	15	4.47 ± 0.47	<0.0001	1.693 ± 0.12
	200	14	9.46 ± 0.78	<0.0001	1.786 ± 0.14
			P=0.000		
19.5 Weeks					
Corn Oil		15	1.90 ± 0.15		2.093 ± 0.12
Benzene	25	15	4.23 ± 0.41	<0.0001	2.773 ± 0.23
	50	15	7.70 ± 0.56	<0.0001	3.233 ± 0.23
	100	15	11.27 ± 0.61	<0.0001	2.767 ± 0.22
	200	14	14.82 ± 0.68	<0.0001	2.964 ± 0.28
			P=0.000		
27 Weeks					
Corn Oil		15	2.00 ± 0.25		2.513 ± 0.12
Benzene	25	15	4.33 ± 0.37	<0.0001	2.960 ± 0.18
	50	15	8.17 ± 0.50	<0.0001	3.767 ± 0.25
	100	15	11.97 ± 0.80	<0.0001	4.673 ± 0.27
	200	14	18.68 ± 1.18	<0.0001	5.343 ± 0.48
			P=0.000		

TABLE 9
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Following Treatment with Benzene by Gavage for up to 27 Weeks

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs	P Value	PCEs (%)
Female					
6.5 Weeks					
Corn Oil		15	1.43 ± 0.22		2.780 ± 0.09
Benzene	25	15	1.17 ± 0.26	0.7754	2.760 ± 0.19
	50	15	1.43 ± 0.24	0.5000	2.760 ± 0.12
	100	15	1.87 ± 0.19	0.0955	2.720 ± 0.10
	200	15	2.30 ± 0.34	0.0070	2.567 ± 0.13
			P=0.000		
13 Weeks					
Corn Oil		15	0.53 ± 0.13		2.140 ± 0.09
Benzene	25	15	1.23 ± 0.22	0.0020	2.447 ± 0.13
	50	15	2.67 ± 0.32	<0.0001	2.407 ± 0.10
	100	15	2.73 ± 0.32	<0.0001	2.520 ± 0.34
	200	15	3.97 ± 0.37	<0.0001	2.240 ± 0.10
			P=0.000		
19.5 Weeks					
Corn Oil		15	1.57 ± 0.25		2.320 ± 0.19
Benzene	25	15	2.50 ± 0.28	0.0056	2.127 ± 0.17
	50	15	3.63 ± 0.29	<0.0001	2.333 ± 0.14
	100	15	4.47 ± 0.34	<0.0001	2.913 ± 0.19
	200	15	6.47 ± 0.40	<0.0001	2.700 ± 0.18
			P=0.000		
27 Weeks					
Corn Oil		15	1.40 ± 0.20		2.633 ± 0.20
Benzene	25	15	2.43 ± 0.28	0.0019	2.520 ± 0.19
	50	15	2.50 ± 0.18	0.0011	2.627 ± 0.29
	100	15	3.50 ± 0.36	<0.0001	3.133 ± 0.17
	200	15	5.93 ± 0.45	<0.0001	3.120 ± 0.15
			P=0.000		

[a] Study was performed at SITEK Research Laboratories, Inc. The detailed protocol is presented by MacGregor *et al*. (1990).
[b] PCE=polychromatic erythrocyte; NCE=normochromatic erythrocyte
[c] Mean ± standard error
[d] Pairwise comparison with the controls, significant at P≤0.006 (ILS, 1990)
[e] Vehicle control
Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990)

polychromatic erythrocyte (PCE) values were increased significantly in male mice at 19.5 and 27 weeks of exposure, indicating an increase in hematopoietic cell proliferation; the greatest increases were seen at 27 weeks in the 100 and 200 mg/kg groups. Percent PCEs were not significantly altered in female mice, although there was some indication of an increase at 19.5 and 27 weeks of exposure.

DISCUSSION AND CONCLUSIONS

Benzene is considered a known human carcinogen by the NTP and the International Agency for Research on Cancer group one carcinogen (IARC, 1982; NTP, 1986). Exposure to benzene is associated with leukemia in humans (USEPA, 1997). In rodents, benzene-exposure produced lymphoma and increases in the number of tumors in other organs. In general, the rat is less responsive than the mouse to the induction of hematopoietic neoplasia.

In this study, the potential for haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice to develop cancer in response to benzene was examined. Benzene was toxic to the hematologic system, particularly to males, resulting in treatment-related body weight effects and one high-dose (200 mg/kg) male died early. Mean body weights of all dosed groups of male mice were decreased compared to vehicle controls, while mean body weights of female mice were decreased only in the 200 mg/kg group. Mean erythrocyte, leukocyte, and lymphocyte counts were depressed at weeks 13 and 27 in dosed males and females. A treatment-related decrease in thymus weights was observed in male mice.

The incidence of malignant lymphoma was significantly increased in 200 mg/kg males compared to the vehicle controls, and the incidence exceeded the incidences in historical controls (Table E1). Most of the lymphomas were observed at the scheduled sacrifice at 27 weeks. Treatment-related bone marrow atrophy, thymus atrophy, atrophy of the mandibular lymph node and other lymph nodes, and hemosiderin pigmentation in the bone marrow occurred in male mice. In addition, treatment-related pigmentation of the skin of the paw and hematopoietic cell proliferation in the spleen were observed in male and female mice.

Malignant lymphomas were not seen in female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice; other studies have also shown that male mice are more susceptible to benzene-induced cancer than female mice after inhalation exposure. This suggests that benzene may be more clastogenic in males than in females. This hypothesis is supported by the

micronucleus findings (Figure 5). Scoring for normochromatic erythrocytes (NCEs) reflects the induction of micronucleated (MN) erythrocytes during the preceding 5 to 6 weeks of dosing (MacGregor, 1990; Witt, 2000). The lowest concentration at which a significant increase in MN-NCEs occurred was 25 mg/kg in male and female mice. However, the number of micronuclei formed in males at the carcinogenic dose of 200 mg/kg at 27 weeks was approximately four times that in female mice. The ability of a chemical to induce micronuclei is generally considered to indicate a potential risk for cancer in humans (Shelby, 1988). Although benzene is a well-documented inducer of chromosomal damage in numerous *in vivo* models, it is not active in bacterial gene mutation assays (Zeiger and Haworth, 1985), likely due to the complex pathways required for biotransformation of benzene to its genetically active metabolites.

Hematopoietic stem cells are thought to be the target for benzene-induced DNA damage and are a small population of the cells in the bone marrow (approximately <0.05%; Morrison and Weissman, 1994); there is some evidence in a Swiss Webster mouse cell culture study that cells from males are more susceptible to benzene than cells from females (Corti and Snyder, 1998). Using cultured hematopoietic stem cells from male and female 129/SvJ mice, it has been shown that there are gender-specific gene expression patterns after benzene exposures (Faiola *et al.*, 2004). Male mice (129/Sv background) deficient in epoxide hydrolase are not as susceptible to benzene induced toxicity as the wild type strain (129/Sv) (Bauer *et al.*, 2003).

Studies have been done to compare gene expression patterns in bone marrow cells derived from $p53^{+/+}$ C57Bl/6 mice versus $p53^{+/-}$ C57Bl/6 mice (Boley *et al.*, 2002). These studies showed that there were significantly higher levels of p21, gadd45, and cyclin G, genes involved in restraining cancer cell growth and repairing DNA damage, after benzene exposure in the $p53^{+/+}$ mice than in the $p53^{+/-}$ mice.

In the benzene study in the $p53^{+/-}$ mouse (Recio *et al.*, 2006), as in the current study, the thymus was a target organ. The $p53^{+/-}$ male mice exposed to benzene by inhalation at 100 ppm 6 hours per day, 5 days a week for up to 52 weeks began to develop thymic lymphomas after 26 weeks of exposure. At the end of the 52 week exposure

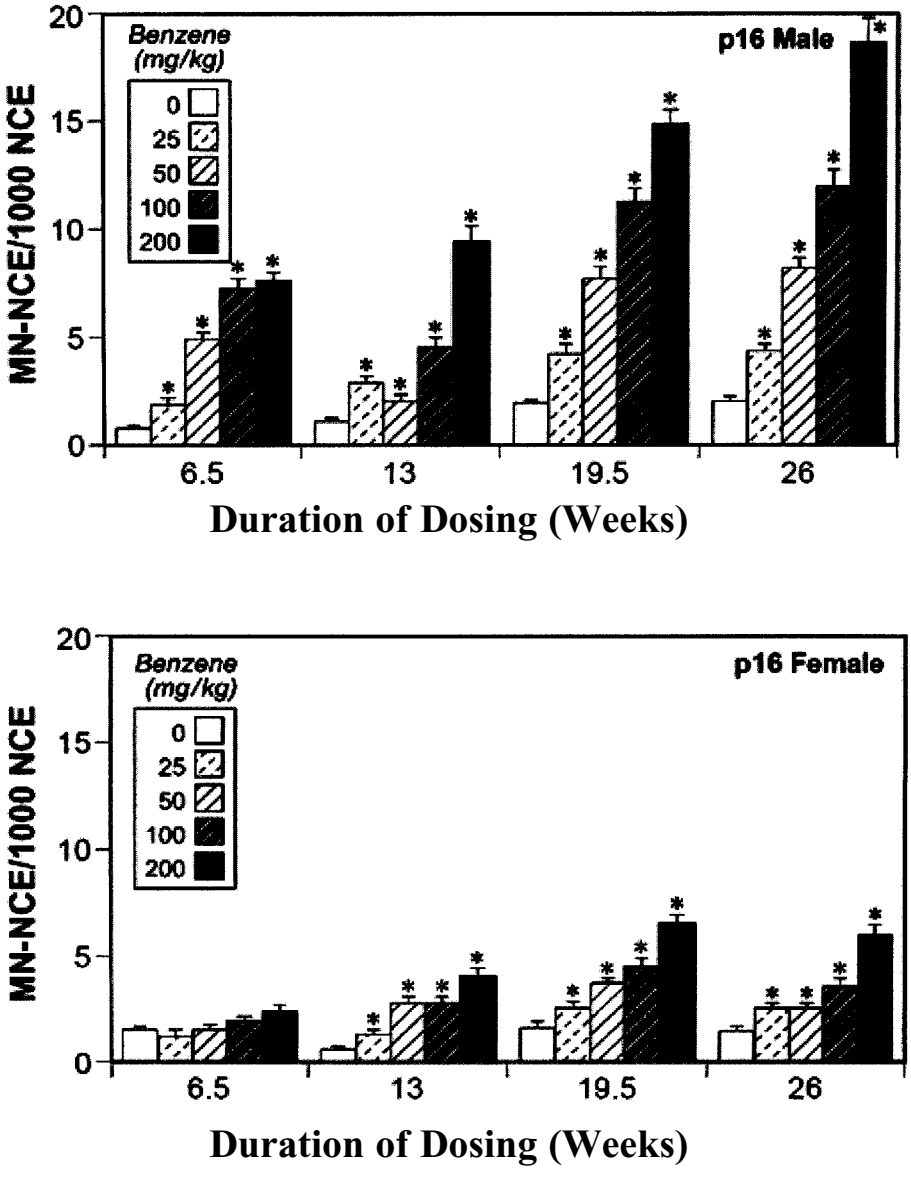


FIGURE 5
Effects of Benzene on the Micronuclei of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice

period, 71% of exposed (100 ppm, 5 days/week, 6 hours/day) $p53^{+/-}$ male mice developed thymic lymphomas versus a less than 10% incidence for these tumors in control $p53^{+/-}$ male mice (Recio *et al.*, 2006). When $p53^{+/-}$ male mice were exposed to benzene by corn oil gavage at 0, 100, or 200 mg/kg for 26 weeks, treatment-related sarcomas and thymic lymphomas occurred (French *et al.*, 2001).

Pigmentation consistent with melanin occurred in males and females at 50, 100, and 200 mg/kg. It is not certain how benzene causes this effect. However, in humans, the p16 (INK4) gene deficiency is often found in melanoma tissue (Sotillo *et al.*, 2001; Soto, 2005). Mice with melanocyte-specific expression of H-*ras* on an INK4a deficient background develop spontaneous cutaneous melanomas (Chin *et al.*, 1997), and INK4a/Arf deficiency promotes ultraviolet-radiation induced melanomagenesis (Recio *et al.*, 2002). Thus, in these benzene studies, the deficiency in the p16 gene might account for the pigmentation observed in melanocytes. However, p16 gene deficiency does not appear to be sufficient for induction of melanomas in mice, but may require deficiency in other genes (e.g., *ras* or p53) (Chin *et al.*, 1997; Bardeesy *et al.*, 2001; Recio *et al.*, 2002; Ackerman *et al.*, 2005).

The haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice strain remains relatively free from neoplasms until 6 to 8 months of age. In the 6-month GMM studies of benzene and phenolphthalein, only one of 30 (3%) of vehicle control haploinsufficient $p16^{Ink4a}/p19^{Arf}$ male mice developed malignant lymphoma, and no neoplasms were found in any other male or female mice (approximate age of mice was 33 weeks) (NTP, 2006b). In 46-week-old mice in the aspartame and glycidol 9-month GMM studies, alveolar/bronchiolar neoplasms, histiocytic sarcoma, and malignant lymphoma occurred in 10% (3/30), 13% (4/30), and 7% (2/30) of vehicle control male mice, respectively, and in 3% (1/30), 47% (14/30), and 0% (0/30) of vehicle control female mice, respectively (NTP, 2005, 2006a).

The current study showed that the haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse had a benzene carcinogenic response after 27 weeks of benzene exposure and that the carcinogenic response was detected with fewer numbers of animals than in the traditional 2-year study in $B6C3F_1$ mice.

The current haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse study detected fewer sites for a benzene carcinogenic response than found in the 2-year $B6C3F_1$ mouse study or in the benzene $p53^{+/-}$ mouse study (Table 1). In addition, the carcinogenic response in the haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse study was only seen in the 200 mg/kg males, while a carcinogenic response occurred in the 2-year $B6C3F_1$ mouse study at 50, 100, and 200 mg/kg and in the 26 week $p53^{+/-}$ male mouse gavage study at 100 and 200 mg/kg. The total benzene dose administered in the 27-week haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse study at the carcinogenic dose of 200 mg/kg was 37,800 mg benzene/kg body weight, and the total benzene dose administered to mice in the 2-year study at the carcinogenic dose of 25 mg/kg was 18,200 mg benzene/kg body weight.

CONCLUSIONS

Under the conditions of this 27-week gavage study, there was *clear evidence of carcinogenic activity* of benzene in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice based on the occurrence of malignant lymphoma. There was *no evidence of carcinogenic activity* of benzene in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ female mice administered 25, 50, 100, or 200 mg/kg.

Treatment of male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice with benzene was associated with toxicity to the hematopoietic system, lymphoid atrophy, and the accumulation of pigment in the extremities.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 9.

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APPENDIX A
SUMMARY OF LESIONS
IN HAPLOINSUFFICIENT $p16^{Ink4a}/p19^{Arf}$ MICE
IN THE 27-WEEK GAVAGE STUDY OF BENZENE

TABLE A1
Summary of the Incidence of Neoplasms in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Early deaths					
Moribund					1
Survivors					
Terminal sacrifice	15	15	15	15	14
Animals examined microscopically	15	15	15	15	15
Alimentary System					
Liver	(15)	(15)	(15)	(15)	(15)
Stomach, forestomach	(15)	(15)	(15)	(15)	(15)
Cardiovascular System					
Heart	(15)	(15)	(15)	(15)	(15)
Endocrine System					
Adrenal cortex	(15)	(15)	(15)	(15)	(15)
Thyroid gland	(15)	(15)	(15)	(15)	(15)
General Body System					
None					
Genital System					
Epididymis	(15)	(15)	(15)	(15)	(15)
Testes	(15)	(15)	(15)	(15)	(15)
Hematopoietic System					
Bone marrow	(15)	(15)	(15)	(15)	(15)
Lymph node	(11)	(11)	(12)	(9)	(7)
Lymph node, mandibular	(15)	(15)	(15)	(14)	(14)
Lymph node, mesenteric	(15)	(15)	(14)	(15)	(15)
Spleen	(15)	(15)	(15)	(15)	(15)
Thymus	(15)	(15)	(15)	(15)	(15)
Integumentary System					
None					
Musculoskeletal System					
None					
Nervous System					
Brain	(15)	(15)	(15)	(15)	(15)

TABLE A1
Summary of the Incidence of Neoplasms in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Respiratory System					
Lung	(15)	(15)	(15)	(15)	(15)
Special Senses System					
Eye					(1)
Urinary System					
Kidney	(15)	(15)	(15)	(15)	(15)
Systemic Lesions					
Multiple organs[b]	(15)	(15)	(15)	(15)	(15)
Lymphoma malignant					5 (33%)
Neoplasm Summary					
Total animals with primary neoplasms[c]					5
Total primary neoplasms					5
Total animals with malignant neoplasms					5
Total malignant neoplasms					5

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Early deaths					
Moribund					1
Survivors					
Terminal sacrifice	15	15	15	15	14
Animals examined microscopically	15	15	15	15	15
Alimentary System					
Liver	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic	3 (20%)	6 (40%)	1 (7%)	3 (20%)	1 (7%)
Salivary glands				(1)	(1)
Submandibular gland, atrophy					1 (100%)
Submandibular gland, inflammation, chronic				1 (100%)	
Stomach, forestomach	(15)	(15)	(15)	(15)	(15)
Erosion					1 (7%)
Hyperplasia	1 (7%)				3 (20%)
Inflammation, acute				1 (7%)	
Stomach, glandular	(15)	(15)	(15)	(15)	(15)
Mineralization			1 (7%)		
Glands, cyst			1 (7%)	1 (7%)	
Cardiovascular System					
Heart	(15)	(15)	(15)	(15)	(15)
Myocardium, inflammation, chronic		1 (7%)	1 (7%)		
Endocrine System					
None					
General Body System					
None					
Genital System					
Epididymis	(15)	(15)	(15)	(15)	(15)
Granuloma sperm					1 (7%)
Unilateral, hypospermia	1 (7%)				
Testes	(15)	(15)	(15)	(15)	(15)
Germinal epithelium, atrophy				1 (7%)	
Germinal epithelium, syncytial alteration				1 (7%)	1 (7%)
Rete testes, inflammation, granulomatous	1 (7%)				
Unilateral, germinal epithelium, atrophy	1 (7%)				

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Hematopoietic System					
Bone marrow	(15)	(15)	(15)	(15)	(15)
Atrophy				10 (67%)	12 (80%)
Pigmentation, hemosiderin	1 (7%)	13 (87%)	13 (87%)	15 (100%)	14 (93%)
Lymph node	(11)	(11)	(12)	(9)	(7)
Mediastinal, atrophy			2 (17%)	6 (67%)	6 (86%)
Lymph node, mandibular	(15)	(15)	(15)	(14)	(14)
Atrophy				6 (43%)	11 (79%)
Hyperplasia, lymphoid	2 (13%)	1 (7%)	1 (7%)		
Infiltration cellular, polymorphonuclear				1 (7%)	1 (7%)
Lymph node, mesenteric	(15)	(15)	(14)	(15)	(15)
Atrophy	1 (7%)	2 (13%)	2 (14%)	13 (87%)	13 (87%)
Infiltration cellular, polymorphonuclear					1 (7%)
Spleen	(15)	(15)	(15)	(15)	(15)
Hematopoietic cell proliferation		1 (7%)		2 (13%)	8 (53%)
Lymphoid follicle, atrophy				15 (100%)	14 (93%)
Thymus	(15)	(15)	(15)	(15)	(15)
Atrophy				7 (47%)	13 (87%)
Mineralization					1 (7%)
Integumentary System					
Skin	(15)	(15)	(15)	(15)	(15)
Pigmentation		15 (100%)	15 (100%)	15 (100%)	14 (93%)
Musculoskeletal System					
Bone	(15)	(15)	(15)	(15)	(15)
Pigmentation, hemosiderin			1 (7%)		
Nervous System					
None					
Respiratory System					
Lung	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic active	2 (13%)		1 (7%)	3 (20%)	5 (33%)
Alveolar epithelium, hyperplasia, focal			1 (7%)		
Bronchiole, hyperplasia, focal			1 (7%)		
Special Senses System					
None					
Urinary System					
Kidney	(15)	(15)	(15)	(15)	(15)
Casts protein	1 (7%)		2 (13%)	1 (7%)	2 (13%)
Nephropathy	1 (7%)	1 (7%)		2 (13%)	4 (27%)

TABLE A3
Summary of the Incidence of Neoplasms in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Survivors					
Terminal sacrifice	15	15	15	15	15
Animals examined microscopically	15	15	15	15	15
Alimentary System					
None					
Cardiovascular System					
None					
Endocrine System					
None					
General Body System					
None					
Genital System					
Ovary	(14)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		2 (13%)			
Hematopoietic System					
Bone marrow	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	3 (20%)	2 (13%)			3 (20%)
Integumentary System					
None					
Musculoskeletal System					
None					
Nervous System					
None					
Respiratory System					
None					
Special Senses System					
None					

TABLE A3
Summary of the Incidence of Neoplasms in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Urinary System					
None					
Systemic Lesions					
Multiple organs[b]	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	3 (20%)	3 (20%)			3 (20%)
Neoplasm Summary					
Total animals with primary neoplasms[c]	3	3			3
Total primary neoplasms	3	3			3
Total animals with malignant neoplasms	3	3			3
Total malignant neoplasms	3	3			3

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Survivors					
Terminal sacrifice	15	15	15	15	15
Animals examined microscopically	15	15	15	15	15
Alimentary System					
Intestine small, duodenum	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic active				2 (13%)	
Liver	(15)	(15)	(15)	(15)	(15)
Hematopoietic cell proliferation				1 (7%)	1 (7%)
Inflammation, chronic	10 (67%)	6 (40%)	6 (40%)	4 (27%)	11 (73%)
Stomach, forestomach	(15)	(15)	(15)	(15)	(15)
Erosion				1 (7%)	
Hyperplasia	1 (7%)				
Inflammation, chronic			3 (20%)		
Stomach, glandular	(15)	(15)	(15)	(15)	(15)
Mineralization					1 (7%)
Cardiovascular System					
Heart	(15)	(15)	(15)	(15)	(15)
Myocardium, inflammation, chronic					1 (7%)
Endocrine System					
Pituitary gland	(15)	(15)	(15)	(15)	(15)
Infiltration cellular, lymphoid	1 (7%)				
General Body System					
None					
Genital System					
Clitoral gland	(1)				
Pigmentation	1 (100%)				
Ovary	(14)	(15)	(15)	(15)	(15)
Angiectasis					1 (7%)
Cyst	1 (7%)			2 (13%)	2 (13%)
Uterus	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic active					1 (7%)
Inflammation, suppurative			1 (7%)	1 (7%)	1 (7%)
Endometrium, hyperplasia, cystic	12 (80%)	15 (100%)	15 (100%)	15 (100%)	14 (93%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Hematopoietic System					
Lymph node	(13)	(11)	(11)	(12)	(13)
Lumbar, hyperplasia, lymphoid					1 (8%)
Lumbar, pigmentation					1 (8%)
Mediastinal, atrophy			2 (18%)	4 (33%)	2 (15%)
Lymph node, mandibular	(14)	(15)	(15)	(15)	(15)
Atrophy		1 (7%)	2 (13%)	4 (27%)	
Hyperplasia, lymphoid	1 (7%)	4 (27%)	2 (13%)	5 (33%)	3 (20%)
Infiltration cellular, polymorphonuclear	1 (7%)				2 (13%)
Lymph node, mesenteric	(15)	(15)	(15)	(15)	(15)
Atrophy		2 (13%)	3 (20%)	8 (53%)	6 (40%)
Hyperplasia, lymphoid					1 (7%)
Spleen	(15)	(15)	(15)	(15)	(15)
Hematopoietic cell proliferation	5 (33%)	5 (33%)	2 (13%)	3 (20%)	7 (47%)
Infiltration cellular, lymphocyte	1 (7%)				
Lymphoid follicle, atrophy		1 (7%)	1 (7%)		1 (7%)
Thymus	(15)	(15)	(15)	(15)	(15)
Hyperplasia, atypical		1 (7%)	1 (7%)	1 (7%)	1 (7%)
Integumentary System					
Mammary gland	(15)	(15)	(15)	(15)	(15)
Hyperplasia					2 (13%)
Skin	(15)	(15)	(15)	(15)	(15)
Inflammation, granulomatous	1 (7%)		1 (7%)		
Inflammation, suppurative				1 (7%)	1 (7%)
Pigmentation		1 (7%)	8 (53%)	15 (100%)	15 (100%)
Musculoskeletal System					
Bone	(15)	(15)	(15)	(15)	(15)
Fibrosis			1 (7%)	1 (7%)	1 (7%)
Nervous System					
None					
Respiratory System					
Lung	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic active	2 (13%)	2 (13%)			2 (13%)
Alveolar epithelium, hyperplasia, focal	1 (7%)				3 (20%)
Special Senses System					
None					
Urinary System					
Kidney	(15)	(15)	(15)	(15)	(15)
Casts protein	5 (33%)	4 (27%)	5 (33%)	8 (53%)	7 (47%)
Infiltration cellular, lymphoid	1 (7%)				
Infiltration cellular, lymphocyte	1 (7%)				
Nephropathy	3 (20%)		1 (7%)	2 (13%)	1 (7%)

APPENDIX B
CLINICAL PATHOLOGY RESULTS

TABLE B1
Hematology Data for Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
n					
Week 13	15	15	15	15	14
Week 27	15	15	15	15	13
Hematocrit (%)					
Week 13	51.5 ± 0.5	50.2 ± 0.7	47.3 ± 0.6**	44.3 ± 0.6**	40.9 ± 0.9**
Week 27	48.4 ± 0.4	47.1 ± 0.9	44.8 ± 0.5**	43.5 ± 0.7**	36.4 ± 1.3**
Hemoglobin (g/dL)					
Week 13	17.1 ± 0.1	16.4 ± 0.2**	15.4 ± 0.2**	14.6 ± 0.3**	13.4 ± 0.3**
Week 27	16.3 ± 0.2	15.8 ± 0.3	15.2 ± 0.2**	14.4 ± 0.2**	12.1 ± 0.4**
Erythrocytes (10^6/µL)					
Week 13	11.51 ± 0.11	10.74 ± 0.16**	10.04 ± 0.15**	9.14 ± 0.14**	8.31 ± 0.22**
Week 27	10.92 ± 0.12	10.20 ± 0.20*	9.55 ± 0.12**	8.99 ± 0.16**	7.11 ± 0.27**
Reticulocytes (10^6/µL)					
Week 13	2.19 ± 0.20	1.85 ± 0.14	1.57 ± 0.14*	1.42 ± 0.18**	1.38 ± 0.17**
Week 27	1.89 ± 0.15	1.99 ± 0.21	2.61 ± 0.10**	3.16 ± 0.29**	2.99 ± 0.35**
Nucleated erythrocytes (10^3/µL)					
Week 13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00**
Week 27	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)					
Week 13	44.8 ± 0.2	46.7 ± 0.2**	47.1 ± 0.2**	48.6 ± 0.2**	49.4 ± 0.3**
Week 27	44.3 ± 0.3	46.2 ± 0.2**	46.9 ± 0.2**	48.4 ± 0.3**	51.4 ± 0.7**
Mean cell hemoglobin (pg)					
Week 13	14.8 ± 0.1	15.3 ± 0.1**	15.4 ± 0.1**	16.0 ± 0.1**	16.2 ± 0.2**
Week 27	15.0 ± 0.2	15.5 ± 0.1**	15.9 ± 0.2**	16.0 ± 0.1**	17.0 ± 0.2**
Mean cell hemoglobin concentration (g/dL)					
Week 13	33.2 ± 0.2	32.7 ± 0.1	32.7 ± 0.1	32.9 ± 0.3	32.8 ± 0.3
Week 27	33.8 ± 0.4	33.6 ± 0.2	33.9 ± 0.3	33.0 ± 0.1	33.1 ± 0.1
Platelets (10^3/µL)					
Week 13	819.2 ± 20.4	795.9 ± 35.3	857.2 ± 39.5	730.1 ± 26.4*	644.4 ± 32.6**
Week 27	944.6 ± 38.5	909.5 ± 39.9	852.3 ± 37.2	816.8 ± 45.3	784.1 ± 39.7**
Leukocytes (10^3/µL)					
Week 13	4.20 ± 0.42	2.79 ± 0.24*	2.38 ± 0.15**	1.42 ± 0.07**	0.93 ± 0.08**
Week 27	5.25 ± 0.41	3.19 ± 0.27**	2.21 ± 0.18**	1.48 ± 0.15**	0.95 ± 0.06**
Segmented neutrophils (10^3/µL)					
Week 13	0.45 ± 0.05	0.41 ± 0.05	0.43 ± 0.05	0.41 ± 0.03	0.33 ± 0.03
Week 27	1.10 ± 0.14	0.81 ± 0.09	0.81 ± 0.12*	0.60 ± 0.11**	0.52 ± 0.04**
Bands (10^3/µL)					
Week 13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 27	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/µL)					
Week 13	3.70 ± 0.39	2.33 ± 0.19*	1.93 ± 0.12**	0.99 ± 0.06**	0.59 ± 0.07**
Week 27	4.12 ± 0.43	2.35 ± 0.23**	1.38 ± 0.08**	0.87 ± 0.09**	0.42 ± 0.03**
Monocytes (10^3/µL)					
Week 13	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
Week 27	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
Basophils (10^3/µL)					
Week 13	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 27	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)					
Week 13	0.04 ± 0.01	0.03 ± 0.01	0.01 ± 0.00*	0.00 ± 0.00**	0.00 ± 0.00*
Week 27	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00

TABLE B1
Hematology Data for Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female					
n					
Week 13	15	15	15	14	15
Week 27	15	15	15	14	14
Hematocrit (%)					
Week 13	50.1 ± 0.5	50.2 ± 0.6	48.8 ± 0.6	47.4 ± 1.1*	46.7 ± 0.8**
Week 27	45.5 ± 0.5	45.6 ± 0.8	46.0 ± 0.8	44.3 ± 0.8	42.9 ± 0.5**
Hemoglobin (g/dL)					
Week 13	17.0 ± 0.2	16.7 ± 0.2	16.4 ± 0.2	15.8 ± 0.4**	15.7 ± 0.3**
Week 27	15.5 ± 0.2	15.4 ± 0.3	15.5 ± 0.3	14.9 ± 0.3	14.2 ± 0.2**
Erythrocytes (10^6/µL)					
Week 13	10.94 ± 0.12	10.83 ± 0.13	10.52 ± 0.13*	9.92 ± 0.26**	9.80 ± 0.17**
Week 27	10.02 ± 0.11	10.02 ± 0.21	10.04 ± 0.20	9.41 ± 0.17**	9.03 ± 0.11**
Reticulocytes (10^6/µL)					
Week 13	2.03 ± 0.15	1.79 ± 0.16	1.99 ± 0.14	2.18 ± 0.36	1.74 ± 0.19
Week 27	1.33 ± 0.12	1.24 ± 0.07	1.25 ± 0.15	1.57 ± 0.14	1.67 ± 0.14
Nucleated erythrocytes (10^3/µL)					
Week 13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 27	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)					
Week 13	45.8 ± 0.2	46.4 ± 0.2*	46.4 ± 0.1*	47.9 ± 0.2**	47.7 ± 0.2**
Week 27	45.4 ± 0.2	45.6 ± 0.3	45.9 ± 0.2	47.1 ± 0.2**	47.5 ± 0.2**
Mean cell hemoglobin (pg)					
Week 13	15.5 ± 0.1	15.4 ± 0.1	15.6 ± 0.1	16.0 ± 0.1**	16.0 ± 0.1**
Week 27	15.5 ± 0.1	15.4 ± 0.1	15.4 ± 0.1	15.8 ± 0.1*	15.7 ± 0.1*
Mean cell hemoglobin concentration (g/dL)					
Week 13	33.9 ± 0.1	33.2 ± 0.1**	33.7 ± 0.1	33.4 ± 0.1*	33.6 ± 0.2
Week 27	34.1 ± 0.1	33.7 ± 0.1*	33.6 ± 0.1*	33.6 ± 0.1*	33.2 ± 0.1**
Platelets (10^3/µL)					
Week 13	698.3 ± 21.2	618.8 ± 24.2	681.6 ± 22.9	629.6 ± 30.4	623.5 ± 29.1
Week 27	748.3 ± 39.9	743.9 ± 37.6	802.1 ± 42.8	725.5 ± 48.8	703.9 ± 53.2
Leukocytes (10^3/µL)					
Week 13	5.07 ± 0.50	4.84 ± 0.35	3.59 ± 0.22*	3.28 ± 0.16**	3.83 ± 0.31*
Week 27	6.25 ± 0.36	6.17 ± 0.48	4.71 ± 0.29**	3.41 ± 0.24**	3.74 ± 0.34**
Segmented neutrophils (10^3/µL)					
Week 13	0.51 ± 0.06	0.44 ± 0.04	0.41 ± 0.03	0.46 ± 0.04	0.74 ± 0.06*
Week 27	0.71 ± 0.11	1.06 ± 0.21	1.06 ± 0.14	0.79 ± 0.09	0.96 ± 0.19
Bands (10^3/µL)					
Week 13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 27	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/µL)					
Week 13	4.53 ± 0.45	4.35 ± 0.33	3.13 ± 0.19*	2.77 ± 0.15**	3.03 ± 0.26**
Week 27	5.48 ± 0.32	5.03 ± 0.37	3.56 ± 0.18**	2.57 ± 0.21**	2.69 ± 0.22**
Monocytes (10^3/µL)					
Week 13	0.01 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Week 27	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.04 ± 0.02
Basophils (10^3/µL)					
Week 13	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 27	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)					
Week 13	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Week 27	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.01

* Significantly different ($P \le 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \le 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

APPENDIX C
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

Table C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
n	15	15	15	15	14
Necropsy body wt	44.0 ± 1.4	38.3 ± 1.4**	38.0 ± 1.0**	31.7 ± 1.2**	32.4 ± 0.8**
Heart					
Absolute	0.204 ± 0.005	0.191 ± 0.005	0.194 ± 0.006	0.186 ± 0.008	0.183 ± 0.007
Relative	4.684 ± 0.136	5.044 ± 0.165	5.148 ± 0.213	5.888 ± 0.182**	5.652 ± 0.174**
R. Kidney					
Absolute	0.307 ± 0.009	0.322 ± 0.006	0.315 ± 0.008	0.304 ± 0.009	0.302 ± 0.005
Relative	7.006 ± 0.123	8.543 ± 0.280**	8.349 ± 0.207**	9.685 ± 0.279**	9.367 ± 0.193**
Liver					
Absolute	1.940 ± 0.094	1.638 ± 0.062**	1.644 ± 0.045**	1.468 ± 0.077**	1.599 ± 0.034**
Relative	43.893 ± 0.932	42.907 ± 0.937	43.413 ± 0.966	46.087 ± 1.229	49.564 ± 0.871**
Lung					
Absolute	0.251 ± 0.008	0.245 ± 0.006	0.244 ± 0.006	0.236 ± 0.008	0.237 ± 0.006
Relative	5.767 ± 0.211	6.540 ± 0.289*	6.492 ± 0.228*	7.509 ± 0.196**	7.387 ± 0.276**
R. Testis					
Absolute	0.119 ± 0.003	0.116 ± 0.002	0.111 ± 0.002*	0.104 ± 0.003**	0.097 ± 0.002**
Relative	2.734 ± 0.105	3.072 ± 0.083*	2.949 ± 0.074	3.326 ± 0.091**	3.028 ± 0.101
Thymus					
Absolute	0.051 ± 0.005	0.039 ± 0.003**	0.031 ± 0.002**	0.019 ± 0.002**	0.021 ± 0.004**
Relative	1.154 ± 0.078	1.028 ± 0.057	0.821 ± 0.044**	0.587 ± 0.057**	0.654 ± 0.109**
Females					
n	15	15	15	15	15
Necropsy body wt	29.8 ± 1.4	28.5 ± 0.9	29.0 ± 0.5	28.7 ± 1.0	26.8 ± 0.5
Heart					
Absolute	0.156 ± 0.004	0.149 ± 0.003	0.149 ± 0.003	0.152 ± 0.003	0.152 ± 0.003
Relative	5.352 ± 0.208	5.270 ± 0.151	5.159 ± 0.118	5.363 ± 0.154	5.688 ± 0.097
R. Kidney					
Absolute	0.224 ± 0.008	0.227 ± 0.005	0.238 ± 0.003	0.227 ± 0.006	0.231 ± 0.004
Relative	7.631 ± 0.221	8.025 ± 0.148	8.241 ± 0.147	7.972 ± 0.225	8.660 ± 0.203**
Liver					
Absolute	1.381 ± 0.058	1.353 ± 0.038	1.376 ± 0.036	1.327 ± 0.047	1.309 ± 0.027
Relative	46.715 ± 1.151	47.756 ± 1.129	47.528 ± 1.110	46.357 ± 1.114	49.028 ± 1.066
Lung					
Absolute	0.236 ± 0.005	0.232 ± 0.005	0.220 ± 0.005	0.226 ± 0.005	0.219 ± 0.004
Relative	8.069 ± 0.272	8.222 ± 0.235	7.628 ± 0.206	7.951 ± 0.204	8.224 ± 0.182
Thymus					
Absolute	0.038 ± 0.003	0.040 ± 0.002	0.033 ± 0.002	0.032 ± 0.003	0.032 ± 0.001
Relative	1.293 ± 0.072	1.407 ± 0.079	1.155 ± 0.089	1.111 ± 0.072	1.183 ± 0.054

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** $P \leq 0.01$
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX D
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

Procurement and Characterization of Benzene

Benzene was obtained from Sigma Aldrich Chemical Co. (Milwaukee, WI) in one lot (00358CS). Identity and purity analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (RTI) (Research Triangle Park, NC) and the study laboratory, Battelle Columbus Operations (Columbus, OH). Reports on analyses performed in support of the benzene study are on file at the National Institute of Environmental Health Sciences.

Lot 00358CS, a colorless liquid, was identified as benzene by RTI using infrared (IR) spectroscopy and proton nuclear magnetic resonance (NMR) spectroscopy and by the study laboratory using IR spectroscopy. Spectra were in agreement with the structure of benzene, literature spectra (*Aldrich*, 1981,1983,1985), and spectra from a frozen reference standard of the same lot. Representative IR and NMR spectra are presented in Figures D1 and D2.

The purity of lot 00358CS was determined by RTI using gas chromatography (GC) by system A (Table D1) and by the study laboratory using GC by system B. GC by system A indicated one major peak and one impurity peak with an area that was less than 0.1% of the total peak area. The overall purity of lot 00358CS was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored at room temperature (25° C) in amber glass bottles with Teflon®-lined lids, protected from light. Stability was monitored twice during the 27-week study with GC by system B; no degradation of the bulk chemical was detected. GC by system B indicated one major peak with a purity of 99.3% relative to a frozen reference standard of the same lot.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing benzene with corn oil (Spectrum Chemicals and Laboratory Products, Inc., Gardena, CA) to give the required concentrations (Table D2). Because animal room sample analyses indicated probable evaporative loss of benzene from the dose formulations during administration, dose formulations prepared on January 4, 2000, or later were prepared with 5% less corn oil than specified in the original SOP for dose formulation. The dose formulations were stored at room temperature (25° C) in amber glass bottles capped with Teflon®-lined lids for up to 35 days.

Stability studies of a 1.0 mg/mL dose formulation of benzene in corn oil were performed by RTI with GC by system C. Stability was confirmed for up to 35 days for dose formulations stored in the dark in sealed glass bottles at refrigerator temperature (5° C) and for up to 3 hours exposed to air and light under simulated dosing conditions.

Periodic analyses of the dose formulations of benzene were conducted by the study laboratory using GC by system B. During the 27-week study, the dose formulations were analyzed four times and postadministration animal room samples were analyzed once (Table D3). All 12 of the dose formulations analyzed and used in the study were within 10% of the target concentrations; all of the four analyzed animal room samples of the dose formulations were within 15% of the target concentration. Periodic analyses of the corn oil vehicle by the study laboratory demonstrated peroxide concentrations below the acceptable limit of 3 mEq/kg.

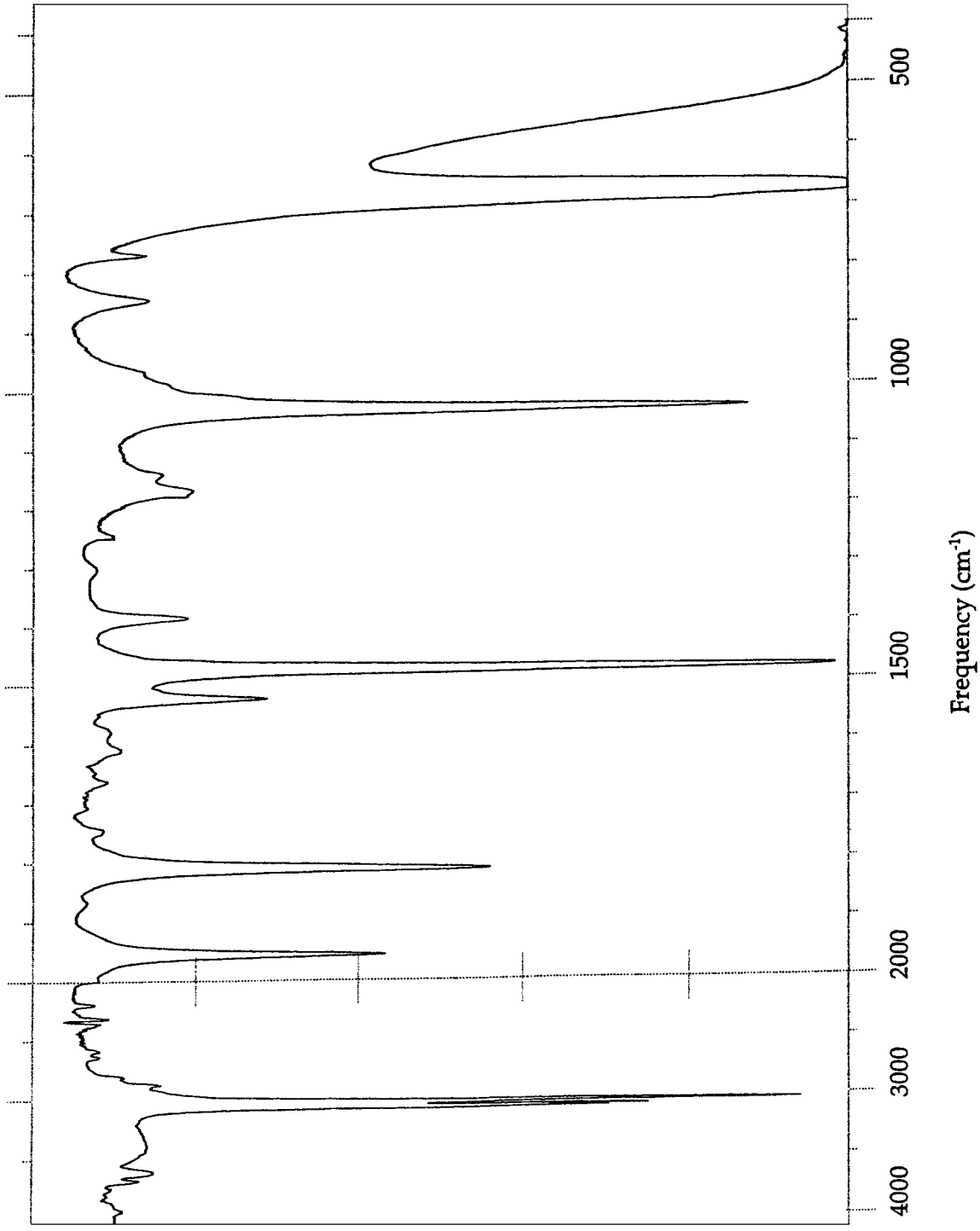

FIGURE D1
Infrared Absorption Spectrum of Benzene

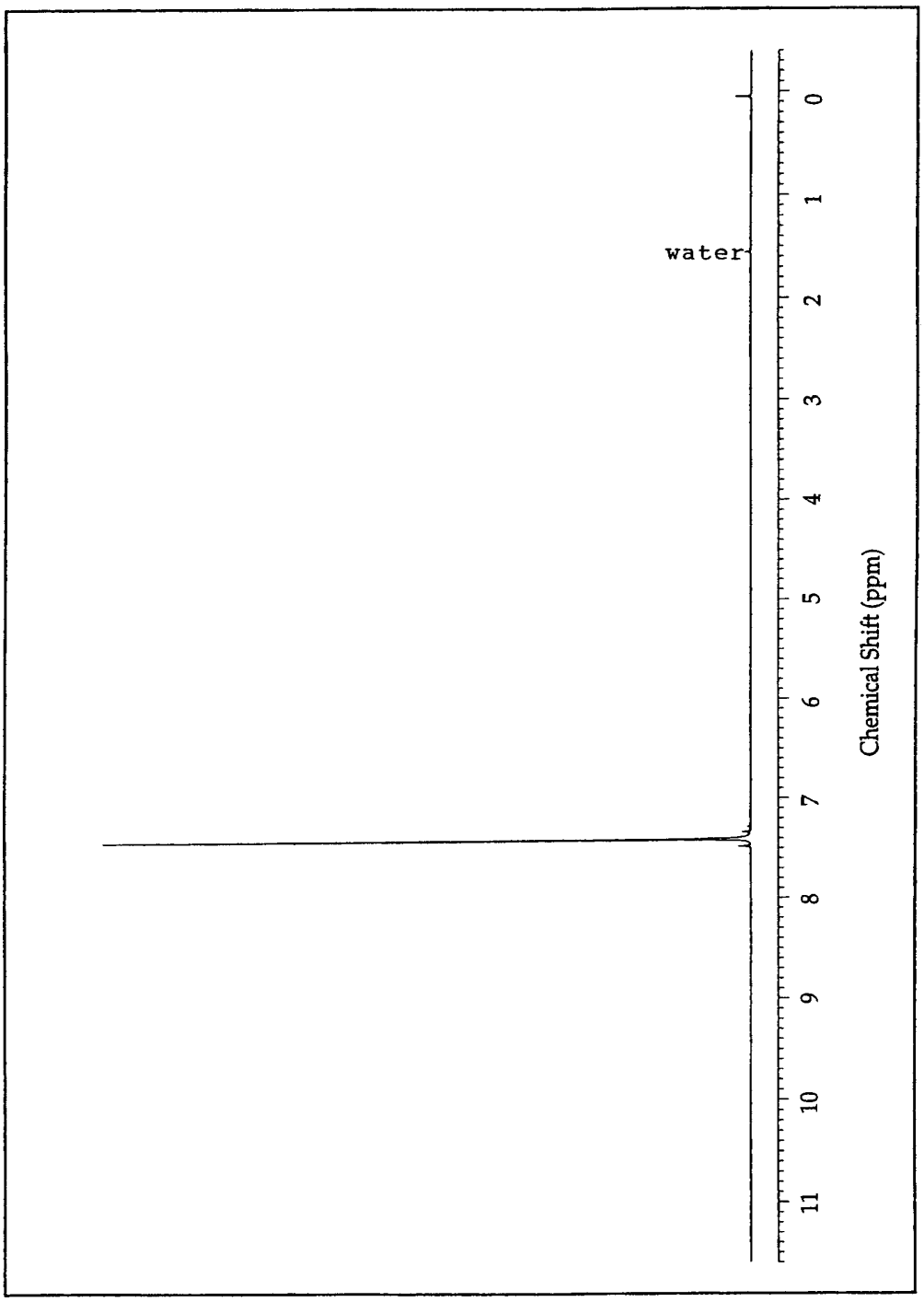

FIGURE D2
Proton Nuclear Magnetic Resonance Spectrum of Benzene

TABLE D1
Gas Chromatography Systems Used in the 27-Week Gavage Study of Benzene[a]

Detection System	Column	Carrier Gas	Oven Temperature Program
System A			
Flame ionization	J&W DB-5, 30 m × 0.53 mm, 5.0-µm film (J&W Scientific, Folsom, CA)	Helium at 2.0 mL/minute	50° C for 5 minutes, then 5° C/minute to 225° C
System B			
Flame ionization	SPB-5, 30 m × 0.53 mm, 3.0-µm film (Supelco, Inc., Bellefonte, PA)	Helium at 4.0 mL/minute	45° C for 3 minutes, then 5° C/minute to 135° C, held for 2 minutes
System C			
Flame ionization	J&W DB-5, 30 m × 0.53 mm, 5.0-µm film (J&W Scientific)	Nitrogen at 4.0 mL/minute	75° C for 3 minutes, then 5° C/minute to 135° C

[a] The gas chromatographs were manufactured by Manufacturer (City, State) (systems A and B) and Hewlett-Packard (Palo Alto, CA) (system C).

TABLE D2
Preparation and Storage of Dose Formulations in the 27-Week Gavage Study of Benzene

Preparation
Formulations were prepared by pipetting the appropriate amounts of benzene into a calibrated mixing bottle containing corn oil, bringing to volume with corn oil, capping, shaking vigorously, stirring on a stir plate for approximately 30 minutes, then shaking vigorously again. To compensate for evaporative loss of benzene from the dose formulations during administration, dose formulations prepared on January 4, 2000, or later were prepared with 5% less corn oil than specified in the original SOP for dose formulation. The dose formulations were prepared approximately every 4 weeks or as needed.

Chemical Lot Number
00358CS

Maximum Storage Time
35 days

Storage Conditions
Dose formulations were stored in amber glass bottles sealed with Teflon®-lined lids at room temperature (25° C).

Study Laboratory
Battelle Columbus Operations, Columbus, OH

TABLE D3
Results of Analyses of Dose Formulations Administered to Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Gavage Study of Benzene

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
October 7, 1999	October 8-9, 1999	2.5	2.352	–6
		5	4.726	–5
		10	9.543	–5
		20	18.47	–8
	November 11-12, 1999[b]	2.5	2.224	–11
		5	4.510	–10
		10	8.541	–15
		20	17.68	–12
December 28, 1999[c]	December 28-29, 1999	2.5	2.229	–11
		5	4.365	–13
		10	8.634	–14
		20	16.09	–20
January 4, 2000[d]	January 4-5, 2000	2.5	2.466	–1
		5	4.834	–3
		10	9.967	0
		20	20.57	+3
March 20, 2000	March 22-23, 2000	2.5	2.443	–2
		5	5.040	+1
		10	9.602	–4
		20	19.89	–1

[a] Results of duplicate analyses. Dosing volume=10 mL/kg; 2.5 mg/mL=25 mg/kg, 5 mg/mL=50 mg/kg, 10 mg/mL=100 mg/kg, 20 mg/mL=200 mg/kg
[b] Animal room samples
[c] Remixed; not used in study
[d] Results of remix. To compensate for evaporative loss of benzene, dose formulations mixed on this date or later were prepared with 5% less corn oil than specified in the original SOP for dose formulation.

APPENDIX E
HISTORICAL CONTROL INCIDENCES

TABLE E1
Historical Incidences of Neoplasms in Control Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Adenoma or Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Histiocytic Sarcoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Malignant Lymphoma	0/15 (0%)	1/15 (7%)	1/30 (3%)
	Aspartame	**Glycidol**	**Total**
40 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	0/15 (0%)	1/15 (7%)	1/30 (3%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	2/15 (13%)	2/30 (7%)
Alveolar/Bronchiolar Adenoma or Carcinoma	0/15 (0%)	3/15 (20%)	3/30 (10%)
Histiocytic Sarcoma	2/15 (13%)	2/15 (13%)	4/30 (13%)
Malignant Lymphoma	0/15 (0%)	2/15 (13%)	2/30 (7%)

[a] Data as of July 6, 2006

TABLE E2
Historical Incidences of Nonneoplastic Lesions in Control Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Kidney			
Casts Protein	1/15 (7%)	0/14 (0%)	1/29 (3%)
Nephropathy	1/15 (7%)	6/14 (43%)	7/29 (24%)
Liver			
Inflammation, Chronic	3/15 (20%)	11/15 (73%)	14/30 (47%)
Spleen			
Hematopoietic Cell Proliferation	0/15 (0%)	2/14 (14%)	2/29 (7%)
Adrenal Cortex			
Subcapsular Hyperplasia	0/15 (0%)	0/14 (0%)	0/29 (0%)
Lung			
Inflammation	0/15 (0%)	0/15 (0%)	0/30 (0%)
Inflammation, Chronic	2/15 (13%)	0/15 (0%)	2/30 (7%)
Stomach, Glandular			
Mineralization	0/15 (0%)	0/14 (0%)	0/29 (0%)
Muscularis Mineralization	0/15 (0%)	0/14 (0%)	0/29 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

TABLE E2
Historical Incidences of Nonneoplastic Lesions in Control Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice

	Aspartame	Glycidol	Total
40 Weeks			
Kidney			
Casts Protein	0/15 (0%)	0/15 (0%)	0/30 (0%)
Nephropathy	2/15 (13%)	4/15 (27%)	6/30 (20%)
Liver			
Inflammation, Chronic	0/15 (0%)	6/15 (40%)	6/30 (20%)
Spleen			
Hematopoietic Cell Proliferation	0/15 (0%)	4/15 (27%)	4/30 (13%)
Adrenal Cortex			
Subcapsular Hyperplasia	2/15 (13%)	1/15 (7%)	3/30 (10%)
Lung			
Inflammation	0/15 (0%)	3/15 (20%)	3/30 (10%)
Inflammation, Chronic	0/15 (0%)	0/15 (0%)	0/30 (0%)
Stomach, Glandular			
Mineralization	0/15 (0%)	10/15 (67%)	10/30 (33%)
Muscularis Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	2/15 (13%)	2/30 (7%)
Myocardium Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

[a] Data as of July 6, 2006

TABLE E3
Historical Incidences of Neoplasms in Control Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Adenoma or Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Histiocytic Sarcoma	3/15 (20%)	0/15 (0%)	3/30 (10%)
Malignant Lymphoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
	Aspartame	**Glycidol**	**Total**
40 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	1/15 (7%)	0/15 (0%)	1/30 (3%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Adenoma or Carcinoma	1/15 (7%)	0/15 (0%)	1/30 (3%)
Histiocytic Sarcoma	5/15 (33%)	9/15 (60%)	14/30 (47%)
Malignant Lymphoma	0/15 (0%)	0/15 (0%)	0/30 (0%)

[a] Data as of July 6, 2006

TABLE E4
Historical Incidences of Nonneoplastic Lesions in Control Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Kidney			
Casts Protein	5/15 (33%)	0/15 (0%)	5/30 (17%)
Nephropathy	3/15 (20%)	10/15 (67%)	13/30 (43%)
Liver			
Inflammation, Chronic	10/15 (67%)	10/15 (67%)	20/30 (67%)
Spleen			
Hematopoietic Cell Proliferation	5/15 (33%)	2/15 (13%)	7/30 (23%)
Uterus			
Endometrium Hyperplasia Cystic	12/15 (80%)	15/15 (100%)	27/30 (90%)
Ovary			
Cyst	1/14 (7%)	1/15 (7%)	2/29 (7%)
Lung			
Inflammation	0/15 (0%)	0/15 (0%)	0/30 (0%)
Inflammation, Chronic	2/15 (13%)	2/15 (13%)	4/30 (13%)
Stomach, Glandular			
Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Muscularis Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

TABLE E4
Historical Incidences of Nonneoplastic Lesions in Control Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice

	Aspartame	Glycidol	Total
40 Weeks			
Kidney			
Casts Protein	0/15 (0%)	0/15 (0%)	0/30 (0%)
Nephropathy	1/15 (7%)	12/15 (80%)	13/30 (43%)
Liver			
Inflammation, Chronic	1/15 (7%)	11/15 (73%)	12/30 (40%)
Spleen			
Hematopoietic Cell Proliferation	5/15 (33%)	9/15 (60%)	14/30 (47%)
Uterus			
Endometrium Hyperplasia Cystic	14/15 (93%)	13/15 (87%)	27/30 (90%)
Adrenal Cortex			
Subcapsular Hyperplasia	14/15 (93%)	15/15 (100%)	29/30 (97%)
Ovary			
Cyst	0/15 (0%)	4/15 (27%)	4/30 (13%)
Lung			
Inflammation	0/15 (0%)	8/15 (53%)	8/30 (27%)
Inflammation, Chronic	0/15 (0%)	0/15 (0%)	0/30 (0%)
Stomach, Glandular			
Mineralization	0/15 (0%)	4/15 (27%)	4/30 (13%)
Muscularis Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Mineralization	0/15 (0%)	1/15 (7%)	1/30 (3%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

[a] Data as of July 6, 2006